The Two Mono-Carbonyl Curcumin Analogs, PGV-1 and CCA-1.1: The Chemopreventive Activity Against DMH-Induced Colorectal Cancer Rat and Proteins Target Candidate Involved

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Research Article

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Abstract

Pentagamavunone-1 and its newest derivatives, CCA-1.1, possess an outstanding cytotoxic activity against several cancer cell lines, especially colorectal cancer. We are continuing to explore the anti-colorectal cancer properties of PGV-1 and CCA-1.1 against DMH-induced colorectal cancer rats and expound the potential protein target in colorectal adenocarcinoma. We utilized DMH 60 mg/kg (subcutaneous injection once a week for 16 weeks) to induce colorectal cancer. PGV-1 and CCA-1.1 at 10 and 20 mg/kg (orally twice a week for 16 weeks) were co-administered with DMH. The WBC count increased in a single DMH group and was countered by co-administration of PGV-1 and CCA-1.1, but no significant differences in RBC. Single DMH treatment for 16 weeks resulted in 80% adenocarcinoma. In contrast, co-administration with PGV-1 and CCA-1.1 suppressed most of the carcinogenic characteristics and symptoms of the pre-malignancy condition. Furthermore, bioinformatics analysis showed that CCA-1.1 has more specific targets than PGV-1, including CDK1, CDK2, MMP3, MMP14, and CYP3A4, which regulate the cell cycle arrest, cancer cell migration, and xenobiotic metabolism, respectively. Interestingly, CCA-1.1 targets CYP3A4, which possibly interrupts DMH metabolism and prevents the initiation of DMH-colorectal carcinogenesis. In conclusion, CCA-1.1 performed better chemopreventive effects against DMH colorectal cancer and might replace PGV-1 to be promoted as a more effective anti-colorectal cancer agent.

1. Introduction

Colorectal adenocarcinoma (COAD) displays a high mortality risk despite the grownup technology for diagnosis and treatment [1]. Chemotherapy remains the furthermost preference due to several inconveniences and obstacles of surgery and radiation to treat accurately at the cancer site [2]. However, the conventional chemotherapeutic drugs for colorectal cancer such as 5-FU, paclitaxel, and epirubicin possess numerous threats of side effects, recurrence, and metastasize induction, which subsequently impacts on patient’s quality of life and survival [3]. Hence, an alternative or new therapeutic agent that is more effective, comfortable, and less toxic to patients is urgently needed to improve colorectal cancer therapy.

Curcumin analog has been extensively studied for its anticancer activity in vitro and in vivo [4]. Two kinds of monocarbonyl curcumin analogs, Pentagamavunone-1 (PGV-1) and Chemopreventive Curcumin Analog 1 (CCA-1.1) (Fig. 1A), were favorable as successor drug for colorectal cancer in several ways: (1) exhibiting irreversible cytotoxic effect in vitro against various cancer cell lines at a lower dose; (2) performing unique target on mitotic phase in the cell cycle; (3) effectively induce cellular senescence and death; (4) suppressing cancer migration in vitro culture system; and (5) inhibiting tumor growth in a xenograft mouse model of leukemia and breast cancer [5–11]. Both compounds exhibit remarkable cytotoxic activities in various mechanisms such as apoptosis, senescence, autophagy, mitotic arrest, and ROS level generation on colorectal cancer cells. Those data give us a foundation for further exploring its mechanism in vivo system.
We currently conducted an in vivo experiment of PGV-1 compared to CCA-1.1 against the DMH-induced colorectal cancer rat model. We observed the cancer incidences, possible safety of the compound based on the body weight, blood profiles, and histopathological appearances. In addition, we also examined the cytotoxic activity of these compounds in vitro using two kinds of colorectal cancer cells, as well as exploration of the potential protein target candidate in COAD using bioinformatics analysis. Hopefully, these results will provide meaningful data on the potency of PGV-1 and CCA-1.1 to develop as colorectal cancer drug candidates, and more specifically, we could determine the better suitable candidate of the two compounds.

2. Materials And Methods

2.1 Chemicals

PGV-1 and CCA-1.1 were obtained from the collection of CCRC, Faculty of Pharmacy UGM, Indonesia.

2.2 Cells Culture

Caco-2 cells were maintained in RPMI medium; CT-26, RAW 264.7, and NIH-3T3 were maintained in DMEM medium (37°C in a 5% CO₂) (Gibco, Invitrogen, USA). Both mediums are supplemented with 10% v/v FBS (Sigma, St. Louis, CA, USA), 150 IU/mL penicillin, and 150 µg/mL streptomycin (Gibco, Invitrogen, USA).

2.3 MTT assay

Cells (10^4 cells/well) were seeded into each well of 96-well plates and treated with various concentrations of CCA-1.1 and PGV-1 for 24 h. After incubation, the media were discharged, and cells were washed with phosphate-buffered saline, then added the MTT reagent (Sigma, St. Louis, CA, USA) and incubated for 4 h. Stopper reagent was added and incubated overnight, and then, the absorbances were measured on a microplate reader (Bio-Rad) under λ = 595 nm.

2.4 Animals and treatment schedules

Thirty-six male Wistar rats (150 – 200 g) were obtained from Gadjah Mada University animal house, divided into 6 treatment groups (Fig. 2A): (I) untreated; (II) DMH and Na-CMC 0.5% (colorectal cancer group); and treatment group of co-administration of DMH with: (III) PGV-1 10 mg/kg BW, (IV) PGV-1 20 mg/kg BW, (V) CCA-1.1 10 mg/kg BW and (VI) CCA-1.1 20 mg/kg BW. The body weight was recorded every week. Animals were sacrificed two weeks after all treatments were completed and the colon was collected. Red blood count (RBC) and white blood count (WBC) were measured using the Automatic Hematology Analyzer Sysmex KX-21. The nodules were recorded for analysis using equation 1.

\[ \text{VN (mm}^3) = \{\text{NL(NW)}^2 \times 0.52\} \]

where VN = volume of nodules, NL = nodule length, NW = nodule width.

2.5 Histopathological examination
The colon organs were fixed in 10% Neutral Buffered Formalin Fixatives (Surgipath, Leica). The paraffin-embedded tissues were cut 5 µm in serial sections using a microtome (Leica Microsystems Inc., Buffalo Grove, IL) and mounted to slides. Slides were then de-waxed by standard deparaffinization with xylol and followed by step-by-step rehydration (100, 95, and 70 % ethanol). Hematoxylin & Eosin (HE) staining was performed by core facilities at the Pathological Anatomy laboratories, Faculty of Medicine, Universitas Gadjah Mada.

2.6 Bioinformatic exploration of PGV-1 and CCA-1.1 in COAD

We utilized the SwissTargetPrediction (http://www.swisstargetprediction.ch/) [13] to obtain the target genes of PGV-1 and CCA-1.1. Potential gene targets in COAD were obtained from GeneCards [14]. Venn diagram was used to overlap the genes and classified its protein class through PANTHER v.16 (http://www.pantherdb.org/) [15]. The UALCAN database [16] was used to assess the expression profile of candidate target genes. We used the GEPIA tool [17] to predict the overall survival, using a Kaplan–Meier survival curve, applying the median cut-off and COAD dataset. The gene expression of colon cancer samples was categorized into high expression (with transcripts per million [TPM] values above the median) and low/median expression (with TPM values below the median). We re-create the graph using GraphPad Prism.

2.7 Statistical analysis

Data were presented as mean ± SEM (n = 3). We used GraphPad Prism software v6.0 (GraphPad Software, La Jolla, CA) for statistical analysis and visualization. The parametric data were analyzed using Student’s t-test or one-way analysis of variance (ANOVA) with Tukey’s multiple comparison post hoc test (*p<0.01; *p<0.001; ns: not significant). Then, the Kolmogorov-Smirnov chi-square test was used for non-parametric data.

3. Results

3.1 Cytotoxic effect of PGV-1 and CCA-1.1

CCA-1.1 was more potent on colorectal cancer cells than PGV-1 but less toxic in non-cancerous cells (Fig. 1B, Table 1). In Caco-2 cells, CCA-1.1 exhibited a significantly stronger cytotoxic effect than PGV-1 with the IC$_{50}$ values of 2.8 and 11.2 µM, respectively. CCA-1.1 also displayed a cytotoxic effect in lower IC$_{50}$ value (2 µM) in CT-26 cells. In addition, both compounds were less toxic against NIH-3T3 and RAW 264.7 cells with IC$_{50}$ values more than 50 µM (Fig. 1C, Table 1). CCA-1.1 exhibited selectivity index (SI) values of more than 17 and 25 in Caco-2 and CT-26 cells, respectively. In comparison, PGV-1 displayed the CI value of more than 4 and 15 in Caco-2 and CT-26 cells, respectively. These findings suggest that both compounds have excellent selectivity and are considered safe chemotherapeutic agents, but CCA-1.1 is preferred.
### Table 1

IC$_{50}$ values of CCA-1.1 and PGV-1 on Caco-2, CT-26, NIH-3T3, RAW 264.7, and CHO-K1 cells and its selectivity index (SI)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Caco-2</th>
<th>CT-26</th>
<th>NIH-3T3</th>
<th>RAW 264.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCA-1.1</td>
<td>2.8</td>
<td>2</td>
<td>&gt; 50</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>PGV-1</td>
<td>11.2</td>
<td>3.2</td>
<td>&gt; 50</td>
<td>&gt; 50</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Selectivity Index (SI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCA-1.1</td>
<td>PGV-1</td>
</tr>
<tr>
<td>Caco-2</td>
<td>&gt; 36</td>
</tr>
<tr>
<td>CT-26</td>
<td>&gt; 50</td>
</tr>
</tbody>
</table>

3.2 Effect of PGV-1 and CCA-1.1 on body weight, growth rate, WBC, RBC, and tumor nodules of DMH-colorectal induced rats

Subcutaneous injection of 60 mg/kg DMH significantly decreased the body weight compared to untreated and other treatment groups (Fig. 2B). Body mass loss was prevented significantly (*p<0.001) on co-administration of DMH with PGV-1 and CCA-1.1, especially at the dose of 20 mg/kg. There is no significant difference in WBC level between DMH + PGV-1/ CCA-1.1 treatment groups and untreated groups (Fig. 2B). CCA-1.1 most effectively countered the WBC elevation caused by DMH at the dose of 20 mg/kg (**p<0.001). For the RBC level, we found no changes at all. Macroscopic examination of single DMH and co-administration of DMH with PGV-1 or CCA-1.1 displayed fluctuating nodule formation in various sizes (Fig. 2C). Colorectal wall thickening was also observed, accompanying nodule formation. In more detail, single administration of DMH induced significantly higher (**p < 0.001) number and volume nodules than DMH + PGV-1/ CCA-1.1 (Fig. 2C). Here, CCA-1.1 at the 20 mg/kg dose was also performed as the most effective in inhibiting tumor development. These findings give us more detailed information that PGV-1 and CCA-1.1 could inhibit tumor progression in DMH-induced colorectal cancer rats with chemoprotective properties.

3.3 Effect of PGV-1 and CCA-1.1 on adenocarcinoma, aberrant crypt, and colitis incidence
Since we used DMH as a colon carcinogen that exhibited multistep carcinogenesis involving a series of pathological alterations [18], we then analyzed the incidence of adenocarcinoma, aberrant crypt, and colitis-associated colorectal cancer of all treatment groups. We could characterize between normal (blue arrow) and multistep of colorectal carcinogenesis as adenocarcinoma (green arrow), aberrant crypts (red arrow), and colitis (yellow square) (Fig. 3A) from DMH-induction and how the treatment of PGV-1 and CCA-1.1 could prevent. Single DMH administration induced 80% adenocarcinoma development throughout the colon. Co-administration with PGV-1 at the doses of 10 and 20 mg/kg BW reduced the percentage of adenocarcinoma (incidence/ group) with the value of 20 and 40%, respectively (Fig. 3B). There is no adenocarcinoma formation on rats administered with DMH + CCA-1.1 at both doses. However, an early characteristic of colorectal carcinogenesis as the aberrant crypt and colitis-associated colorectal cancer in DMH + PGV-1/ CCA-1.1 groups was higher than the single DMH group. At the 20 mg/kg BW dose, CCA-1.1 is more efficient in inhibiting colorectal carcinogenesis than PGV-1 at the same doses. We believe that adenocarcinoma formation is always accompanied by a multi-combination of aberrant crypts, colitis, and inflammation [19]. Still, we have not counted it as an individual case of aberrant crypts or colitis and only focused on the representative pathological incidence of each colon/ rat to evaluate the carcinogenic step of DMH induction. Overall, both PGV-1 and CCA-1.1 could suppress the carcinogenesis of DMH-induction in rats, particularly CCA-1.1.

3.4 Bioinformatic exploration of PGV-1 and CCA-1.1 on COAD

For further comprehension of PGV-1 and CCA-1.1 targets in COAD, bioinformatic analysis elucidate that CCA-1.1 targets more genes than PGV-1 (Fig. 4A). The sixteen CCA-1.1 target genes were classified into a variety and rich protein classes than the five target genes of PGV-1 (Fig. 4B). We then emphasize the five genes which are specifically involved in cancer metabolism (CYP3A4), cell proliferation (cell cycle regulator) (CDK1 and CDK2), and cell migration (MMP3 and MMP14) as the potential target of CCA-1.1. We found that CDK1, CDK2, MMP3, and MMP14 were significantly overexpressed in tumors than in normal colon tissues (*p<0.01; **p<0.0001) (Fig. 4C). CYP3A4 was higher expressed in normal tissue than in the tumor, confirming that this gene plays a role as a xenobiotic metabolizer to counter the pathological cascade [20, 21]; in this case, DMH-carcinogenesis. On the other hand, the overall survival was not significantly different between high and low expression of those respective genes for 150 months span (Fig. 4C). Although not statistically different, COAD patients who had increased MMP14 and CYP3A4 intended to have a higher risk of relapse than patients with lower expression. By looking at this information, we believed that CCA-1.1 target genes in these results were unique and interesting points to explore and strongly correlated with in vitro and in vivo experiments.

4. Discussion
Formerly, PGV-1 has been shown anti-tumorigenic activities against leukemia and breast cancer in xenograft mice model [6, 7], but has not been explored in colorectal cancer. While the other curcumin analogs, pentagamavunone-0 (PGV-0), gamavutone-0 (GVT-0), and hexagamavunone-0 (HGV-0), exhibited anti-inflammatory activity in the DMH-colorectal cancer rat model [12]. In this regard, we challenge and continue to explore PGV-1 and CCA-1.1, a targeted and selective candidate for colorectal cancer chemotherapeutic agents through \textit{in vitro}, \textit{in vivo}, and bioinformatic exploration.

In the \textit{in vitro} study, we confirm that CCA-1.1 was more potent than PGV-1 and underlined with a superior safety level. Caco-2 and CT-26 colorectal cancer cells represent the COX-2 level in colorectal cancer since there was fluctuate expression of COX-2 in COAD patients [22, 23]. The different characteristics of the two cells that we used provide different responses for each cell. We found compelling evidence that PGV-1 cellular response, in this case, cytotoxic activity, might be altered by COX-2 expression. Further confirmation on the selectivity index of CCA-1.1 and PGV-1 indicate that both compounds are promising candidates of selective anticancer agents. It was also confirmed by the WBC and RBC level in the \textit{in vivo} experiment that PGV-1 and CCA-1.1 might defeat tumor formation with fewer or no observable opposing effects on the normal lineage of cells. The RBC and WBC profiles could explain a slight pathological condition of colorectal cancer, such as anemia, crur, and inflammation [1, 24]. The safety properties of CCA-1.1 and PGV-1 are important to develop new anticancer agents. However, this study is still limited in the normal proliferating immortal cells that should be evaluated in the normal primary cells as well as in the \textit{in vivo} model.

We performed the chemopreventive examination of PGV-1 and CCA-1.1 that simultaneously administered with DMH for inducing colorectal cancer, whether they were able to reverse, suppress, or prevent the initial phases of DMH carcinogenesis. We believed that the study of \textit{in vivo} colorectal carcinogenesis had a remarkably long journal accompanying the development of diagnostic, pathway mechanism, and therapy of cancer [25]. For the inducing agent, we used DMH as a procarcinogen that metabolizes to a methyl free radical and generates hydroxyl radicals of metal ions resulting in lipid peroxidation, molecular genetic alteration, and cancer initiation [26]. We found that co-administration of PGV-1 and CCA-1.1 in both doses (10; 20 mg/kg) with DMH were adequate to suppress tumor growth with noticeable toxic effects to the normal lineage cells caused by DMH, particularly CCA-1.1 at 20 mg/kg. The mechanism may, in part concern with the \textit{in vitro} anticancer activities of PGV-1 and CCA-1.1, including induction of apoptosis, targeted cell cycle arrest, related cancer marker protein inhibition, and selective on cancer cells [7, 8]. Then, PGV-1 and CCA-1.1 may play a role as free radical eliminators through enzymatic antioxidants since curcumin analogs are known as dual-oxidant on their anticancer features [27]. This phenomenon should be an exciting point to further exploration related to their antioxidant activities.

Bioinformatic exploration was confirmed that CCA-1.1 was more potent than PGV-1. The proteins target candidate of CCA-1.1 in COAD was richer than PGV-1, validating previous \textit{in vitro} observation of CCA-1.1 that report several activities such as apoptosis, anti-migration/ metastasis, and specific G2/M cell cycle arrest [11, 28–32]. We underlined the two proteins CDK1 and CDK2, which play a specific role in the G1/S and G2/M cell cycle stage, respectively [33]. Those two proteins might become an essential protein in the
anticancer effect of CCA-1.1 in the field of cell proliferation and cell cycle regulation on colorectal cancer. On the other hand, targeting MMP3 and MMP14 [34] might also underline the better effect on anti-migration of CCA-1.1 than PGV-1. CCA-1.1 targets CYP3A4 should be highlighted as superior to PGV-1. By targeting this protein, CCA-1.1 possibly suppressed or inhibited DMH-carcinogenesis since DMH is mainly metabolized into toxic metabolites by CYPs in the liver, including CYP3A4 [20, 21]. We believed that this finding could emphasize the superiority of CCA-1.1 compared to PGV-1 to develop as a colorectal cancer chemotherapeutic candidate. An additional laboratory experiment is required for a better and detailed comprehension of the mechanism.

Since we used the DMH-induced colorectal cancer model, we could also observe an early pre-neoplastic hyperproliferative lesion that formed aberrant crypt foci (ACF) as intermediary indicators of carcinogenesis [35]. Here, we focused on the late and early stages of colorectal carcinogenesis to define the effect of suppression or inhibition of PGV-1 and CCA-1.1. We found that single DMH induced late stage of colorectal cancer, which is adenocarcinoma with severe alteration of goblet cell's structure, inflammation, and invasive growth of cancer cells. However, co-administration of DMH with PGV-1/ CCA-1.1 induced an early stage of colorectal carcinogenesis, such as aberrant crypt [36] and colitis-associated colorectal cancer (severe inflammation and hemorrhagic) [37], without adenocarcinoma formation, especially CCA-1.1 at both doses. These findings indicated that PGV-1 and CCA-1.1 could suppress or inhibit the carcinogenesis of DMH in rats. It should also be superior to a new anti-colorectal cancer due to its solubility, stability, and effectiveness, especially in an acidic environment since both compounds were administered orally to rats. However, several conventional chemotherapeutic drugs are still limited for oral use due to their stability in the alimentary tract [38–40]. In this study, we used a chemopreventive setting on the treatment schedule, in which DMH was co-administered with PGV-1/ CCA-1.1 at the same period. Further exploration using another treatment design would be great to establish the detailed mechanism of those compounds and the possibility to pharmaceutically developed as orally administered drugs for colorectal cancer.

To our knowledge, it is the first time that in vivo exploration of PGV-1 and CCA-1.1 against colorectal cancer has been conducted along with in vitro and bioinformatic exploration. These findings revealed a great potential of PGV-1 and CCA-1.1 to develop as anti-colorectal cancer agents. However, the optimum dose to give as well as the treatment setting may not be answered yet. A deeper investigation is needed to settle down the potency of PGV-1 and CCA-1.1 as anti-colorectal cancer agents.

5. Conclusion

Oral co-administration of PGV-1 and CCA-1.1 (10 and 20 mg/kg) for 16 weeks significantly inhibited colorectal carcinogenesis, compared to single DMH and untreated groups without toxicity in normal lineage cells. The suppressing effect of PGV-1 on COAD development is suggested to target signal transduction and cell cycle, whereas CCA-1.1 targets cell cycle, signal transduction, cell migration, and carcinogen metabolism. CCA-1.1 performed a better chemopreventive effect as a new anti-colorectal cancer candidate against DMH colorectal cancer than PGV-1, possibly supported by the additional target
on CYP3A4. In general, both compounds exhibited strong chemopreventive potential against colorectal cancer.

**Declarations**

**FUNDING**

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**AUTHORS’ CONTRIBUTIONS**


**COMPLIANCE WITH ETHICAL STANDARDS**

*Declaration of interest*

The authors report no conflicts of interest.

*Ethical approval*

This article contain study with animals approved by the Ethics Committee of Gadjah Mada University Indonesia (Number. 00001/04/LPPT/I/2020).

*Consent to participate*

Not applicable

*Consent for publication*

Not applicable

*Availability of data and material*

Not applicable

*Code availability*

Not applicable

**References**


**Figures**

**Figure 1**

(A) Chemical structures of PGV-1 and CCA-1.1.

(B) Cell viability (% vs. Concentration [nM]) graphs for Caca-2 and CT-26 cells treated with CCA-1.1 and PGV-1.

(C) Cell viability (% vs. Concentration [nM]) graphs for NIH-3T3 and RAW 264.7 cells treated with CCA-1.1 and PGV-1.
The cytotoxic effects of PGV-1 and CCA-1.1 against several cell lines. (A) Chemical structure of PGV-1 and CCA-1.1. (B) Growth inhibitory profiles of PGV-1 and CCA-1.1 against Caco-2 and CT-26 colorectal cancer cells. (C) Growth inhibitory profiles of PGV-1 and CCA-1.1 against NIH-3T3 and RAW 264.7 non-cancerous cells.

**Figure 2**
Co-administration of PGV-1 and CCA-1.1 suppressed the carcinogenic symptoms of DMH. (A) Schematic treatment schedule of experiment. (B) Body weight and blood profiles (WBC and RBC) of each treatment group. (C) Macroscopic appearance and nodules calculation (number and volume) of each treatment group. All data presented as mean ± SEM; *p < 0.01, **p < 0.001 compared to DMH-induced colorectal cancer rat.

Figure 3

PGV-1 and CCA-1.1 suppressed most of the pre-malignancy condition of DMH induction. (A) Microscopic examination of colon tissues using H&E. Representative images of the normal tissue, colon adenocarcinoma, aberrant crypt, and colitis appearance (400x). Blue arrows indicate normal crypt architecture. Green arrows indicate destroyed crypts and green square indicate an invasive adenocarcinoma. Red arrows indicate aberrant crypts as early step of colon-adenoma formation. Yellow squares indicate inflammation-associated colitis. (B) % of adenocarcinoma, aberrant crypt, and colitis incidence/ group (n = 6).
Figure 4

Bioinformatic exploration of CCA-1.1 and PGV-1. (A) Target genes of CCA-1.1 and PGV-1 overlapped with colorectal adenocarcinoma (COAD) biomarkers from the GeneCards. (B) The pie chart of CCA-1.1 and PGV-1 target genes classification based on their protein class using PANTHER. (C) Expression and overall survival of several target genes in COAD obtained from the UALCAN website (http://ualcan.path.uab.edu) based on TCGA dataset.
Figure 5

Proposed mechanism of PGV-1 and CCA-1.1 in DMH-colorectal cancer.