ORIGINAL ARTICLE

# Tumour-suppressive effects of curcumin analogs CCA-1.1 and Pentagamavunone-1 in colon cancer: *In vivo* and *in vitro* studies

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## ABSTRACT

This study aimed to evaluate the efficacy of Chemoprevention Curcumin Analog-1.1 (CCA-1.1) and Pentagamavunone-1 (PGV-1) *in vivo* and *in vitro* in colorectal cancer model. CCA-1.1 or PGV-1 was administered orally to 1,2-dimethylhydrazine (DMH)-induced rats for 16 weeks. The cytotoxicity of both compounds was tested on Caco-2, CT26, and NIH/3T3 cells using the MTT method. The cell cycle, apoptosis, and reactive oxygen species (ROS) levels were analyzed through flow cytometry. X-gal staining was used to examine the compound's effect on senescence. Oral co-administration of CCA-1.1 or PGV-1 significantly suppressed the carcinogenic characteristics and symptoms of premalignant colon cancer relative to DMH-only and untreated groups. CCA-1.1 and PGV-1 administration did not affect the blood profile. CCA-1.1 and PGV-1 demonstrated great cytotoxicity on Caco-2 and CT26 cells, with 50% inhibition concentration ($IC_{50}$) values of 4.3 ± 0.2 and 3.1 ± 0.1 μM for CCA-1.1 and 11.2 ± 1.1 and 4.8 ± 0.1 μM for PGV-1, respectively, while not toxic against fibroblast cells. Both compounds instigated G2/M arrest and efficiently induced cell senescence and apoptosis. Moreover, these analogs selectively elevated oxidative stress in colon cancer cells without inducing noticeable changes in fibroblasts. In conclusion, PGV-1 and CCA-1.1 suppressed colorectal tumor formation and induced mitotic arrest.

**Key words:** 1,2-dimethylhydrazine-induced rats, chemopreventive, colorectal cancer cells, curcumin analogs

## INTRODUCTION

A curcumin-based analog compound, namely Pentagamavunone-1 (PGV-1) [Figure 1a], has been synthesized and has potency as chemotherapy drug through several targets in the physiological process of cancer cell proliferation, both *in vitro* and *in vivo*.[1-4] Despite its effectiveness, structural modification of PGV-1 is needed to improve its solubility without diminishing its cytotoxic effect against cancer cells.[5] The carbonyl group of PGV-1 is first changed to a hydroxyl group, and the resulting compound is known as Chemoprevention Curcumin Analog-1.1 (CCA-1.1).[5] CCA-1.1 demonstrates similar anticancer effects as PGV-1 against breast, liver, and leukemia cells.[6-8] Interestingly, CCA-1.1 performs anti-migratory activities

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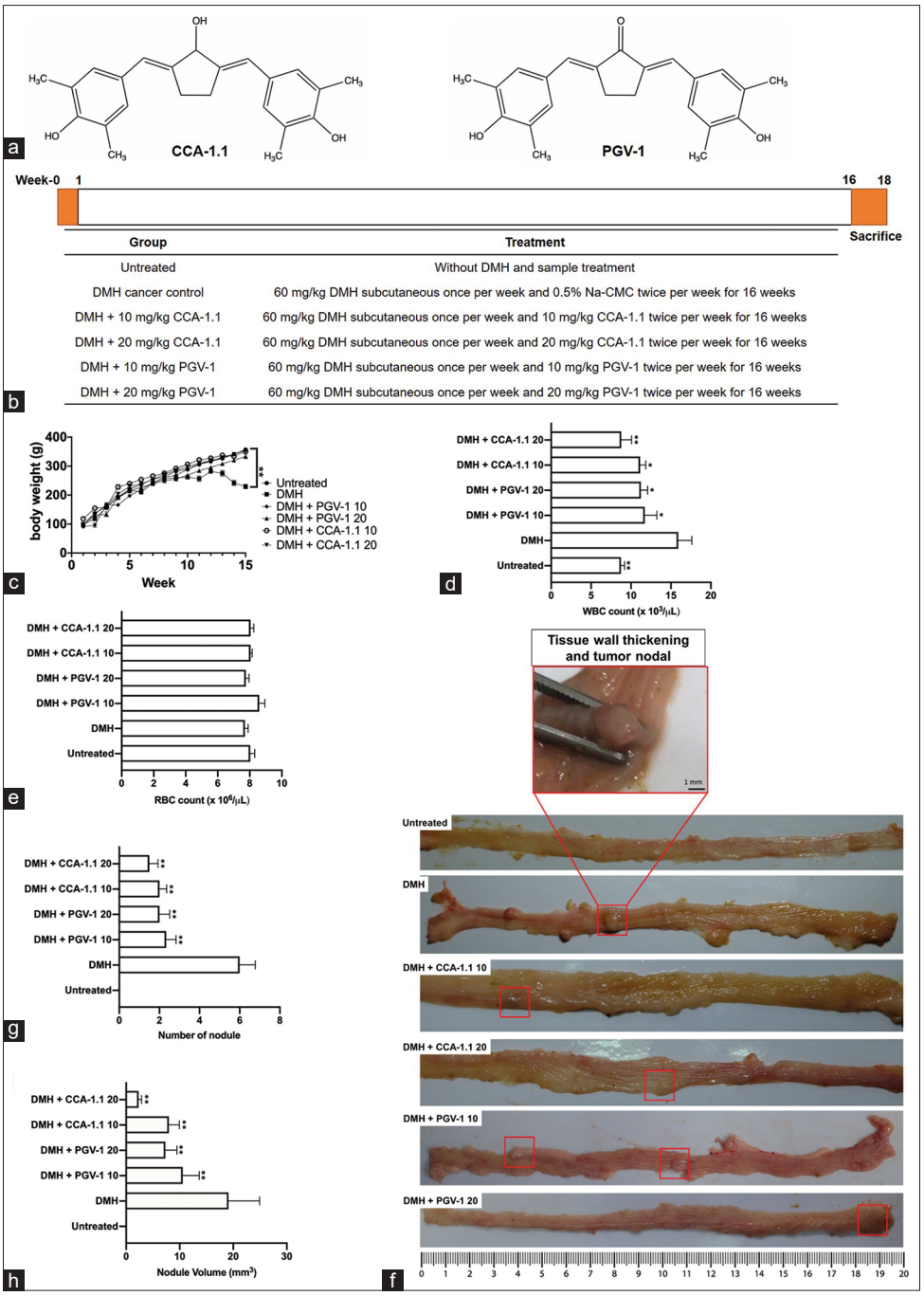



**Figure 1:** Coadministration of Chemoprevention Curcumin Analog-1.1 (CCA-1.1) or Pentagamavunone-1 (PGV-1) delays tumor growth upon 1,2-dimethylhydrazine induction. (a) Chemical structure of CCA-1.1 and PGV-1. (b) Animal experimental design. (c) Rat body weight, (d) white blood count, and (e) red blood count profiles. (f) Representative macroscopic appearances of the colon. The number (g) and volume (h) of nodules in the colon from each treatment group. Data presented as mean ± SD ($n = 6$). *$P < 0.05$; **$P < 0.01$ compared to the DMH-induced group

better compared to PGV-1 in WiDr colorectal cancer cells.[9,10] Thus, CCA-1.1 shows potential to be developed as chemotherapy for colon cancer. *In vivo* studies and further cellular mechanism investigations are needed to confirm the potency of the anti-colon cancer effect of CCA-1.1 as a chemotherapy drug candidate.

Unlike PGV-1 and curcumin, CCA-1.1 has higher solubility in phosphate buffers,[5] thus enabling the use of *in vivo* systems in evaluating its pharmacological effects. Moreover, an *in vivo* protocol for the selective induction of colon carcinoma using rats injected with 1,2-dimethylhydrazine (DMH) has been established, allowing screening for compounds with putative antitumor effects.[11,12] The effect of these compounds on DMH-induced rats will yield valuable information regarding their potential use as oral antitumor drugs for colon cancer therapy. Hence, this study aims to evaluate the antitumor activities of CCA-1.1 and PGV-1

based on their chemopreventive activity in preventing colon tumor formation in DMH-induced rats as well as their effects on the cellular physiology of colorectal cancer cells.

## MATERIALS AND METHODS

### Materials

The curcumin analogs were prepared by Cancer Chemoprevention Research Center, Universitas Gadjah Mada (UGM).[5] CCA-1.1 or PGV-1 was diluted in 0.5% sodium carboxymethyl cellulose (Na-CMC) (Sigma-Aldrich, Singapore) for oral administration. 1,2-Dimethylhydrazine dihydrochloride (DMH) was purchased from Santa Cruz Biotechnology (sc-358719) (USA).

### Animals and the experimental design

All the procedures were granted permission from the Experimental Animal Ethics Board of UGM (reference no. 00001/04/LPPT/I/2020). Wistar rats aged 4 weeks were randomly allocated to one of six treatment groups, as depicted in Figure 1b. Animals were cared for in environmentally controlled conditions. The body weight was recorded every week. Animals were sacrificed 2 weeks after all treatments were completed. Before dissection, the white blood count (WBC) and red blood count (RBC) were measured with hematology analyzer (Sysmex KX-21, Japan).[2] Subsequently, the rat abdominal cavity was dissected, and the colon and rectum were collected, and later washed in the saline solution. The nodule dimensions were determined as previously described.[12]

### Histopathological examination

Extracted colons were stored in 10% formalin buffer (Surgipath, Leica, USA). Three tissue sections randomly chosen per animal were made into paraffin blocks and mounted to slides into a 5 μm section using a microtome (Leica, USA).[12] The slides were stained for hematoxylin–eosin. Histological examination was performed in a blind manner.[13] The presence of adenocarcinoma, aberrant crypt foci, and colitis was calculated for each group and expressed as an incidence proportion per group.

### Cell culture

Caco-2 cells were cultured in RPMI-1640 medium, whilst CT26 and NIH/3T3 cells were cultured in DMEM (Gibco, USA); Both mediums were added with fetal bovine serum (FBS) (Gibco, USA) and antibiotics (Penstrep) (Gibco, USA). All cells were stored at 37°C in a 5% $CO_2$ incubator.

### MTT assay

A total of $10^4$ cells/well were treated with CCA-1.1 or PGV-1 (0.5 – 10 μM in culture medium) for 24 h. Later, the treatment medium was discarded, and 0.5% MTT in fresh medium (Sigma-Aldrich, USA) was inputted into well as previously explained.[9] The absorbance after enzymatic reaction was determined on microplate reader (Bio-Rad, USA).

### Cell cycle and apoptosis analyses

Briefly, $5 \times 10^5$ cells were plated prior tested with CCA-1.1 or PGV-1 for 24 h. The treated cells were collected for subsequent testing as per the manufacturer's manual for cell cycle analysis using Cycletest PlusKit (BD Biosciences, USA) and FITC Annexin V-FLUOS kit (Roche, Switzerland) for apoptosis occurrence. The stained cells were subjected into flow cytometer (Accuri C6, BD Biosciences, USA) for further analysis.

### Intracellular reactive oxygen species measurement

Briefly, $5 \times 10^4$ cells were suspended in 500 μl of 10% FBS in PBS. Cells were incubated with 2′,7′–dichlorofluorescin diacetate (D6883, Sigma-Aldrich, USA) for 30 min (final concentration 20 μM) before treatment with sample for the next 4 h. The level of reactive oxygen species (ROS) was analyzed with flow cytometer.[10]

### Senescence assay

Cells were fixed using a 4% paraformaldehyde solution (Millipore, Germany) for 10 min, then incubated in a staining solution (0.2% X-Gal) (B4252, Sigma-Aldrich, USA) for 72 h. The stained cells were documented under a light microscope.[6]

### Statistical analysis

The statistical analysis was processed with GraphPad Prism for Mac version 9.0 (USA) using analysis of variance and Dunnett's tests for multiple comparisons *post hoc*. All data were analyzed at a 95% confidence level.

## RESULTS

### The chemopreventive activities of Chemoprevention Curcumin Analog-1.1 (CCA-1.1) and Pentagamavunone-1 (PGV-1) *in vivo*

DMH induction significantly suppressed body weight ($P < 0.01$) [Figure 1c]. Body mass loss was prevented by PGV-1 and CCA-1.1, notably at a dose of 20 mg/kg, and CCA-1.1 countered WBC elevation caused by DMH ($P < 0.001$) [Figure 1c and d]. We found no differences in RBC levels among groups [Figure 1e]. PGV-1 or CCA-1.1 administration inhibited DMH-induced nodule formation and thickening of the colon wall [Figure 1f] and also reduced the nodule's number and volume ($P < 0.001$) [Figure 1g and h]. There were no abnormal behaviors in rats throughout the experiment.

The occurrence of adenocarcinomas, aberrant crypt foci, and colitis-associated colorectal malignancies across all treatment groups was investigated [Figure 2a]. These features were distinguished [Figure 2b] in the DMH-induced group. DMH administration alone resulted in 80% adenocarcinoma

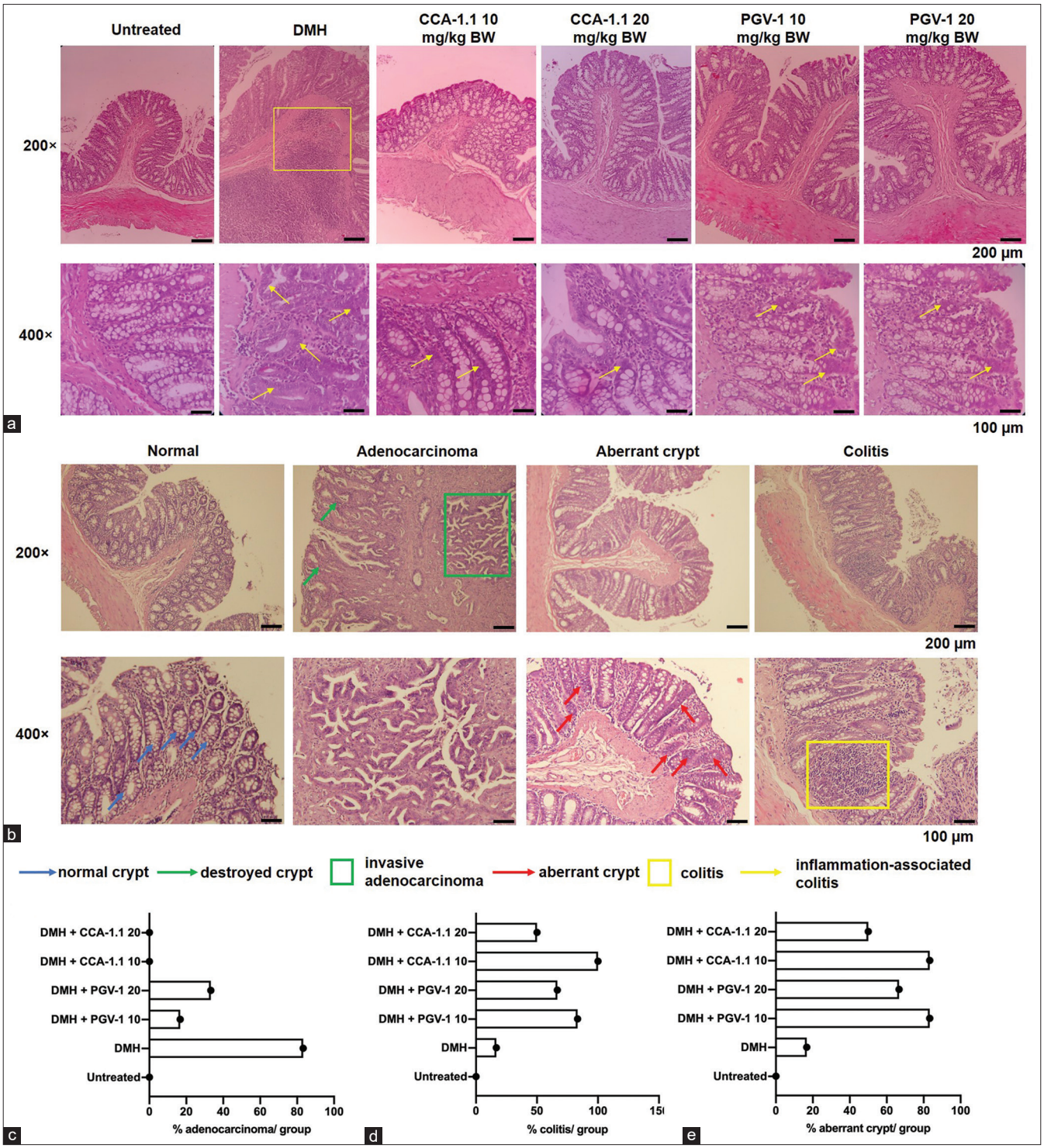



**Figure 2:** Chemoprevention Curcumin Analog-1.1 (CCA-1.1) or Pentagamavunone-1 (PGV-1) suppresses carcinogenic symptoms and most pre-malignancy conditions in dimethylhydrazine1,2--induced rats. (a) Representative images of hematoxylin-eosin-stained colon tissues. (b) Representative images of adenocarcinoma, aberrant crypt, and colitis incidence. Scale bars = 200 and 100 µm, respectively at × 200 and × 400 magnification. Six animals per group were obtained, 3 tissue sections per animal were analyzed randomly. The number of rats with colon adenocarcinoma (c), aberrant crypt (d), and colitis (e) is calculated per total rats in each group

growth, but coadministration with PGV-1 reduced the up to 40% [Figure 2c]. No adenocarcinoma development was observed in rats given DMH + CCA 1.1 at both doses, though the colitis and aberrant crypt were still present [Figure 2d and e]. Thus, both PGV-1 and CCA-1.1 prevented DMH-induced adenocarcinoma progression.

### The cytotoxic activities of CCA-1.1 and PGV-1 *in vitro*

We use the *in vitro* system in colorectal cancer cell lines, Caco-2 and CT26, and noncancerous cells (NIH/3T3). CCA-1.1 revealed stronger cytotoxicity compared to PGV-1 with $IC_{50}$ values of 4.3 ± 0.2 and 11.2 ± 1.1 μM, respectively, in Caco-2 cells [Figure 3a]. Meanwhile, in CT26 cells, CCA-1.1 and PGV-1 exhibited antiproliferative effects at $IC_{50}$ values of 3.1 ± 0.1 μM and 4.8 ± 0.1 μM, subsequently [Figure 3b]. Both compounds were not toxic on NIH/3T3 cells [Figure 3c] and proved selectivity as indicated by the selectivity index values that > 1 [Figure 3d].

### CCA-1.1 and PGV-1 modulate colorectal cancer cell cycle and apoptosis

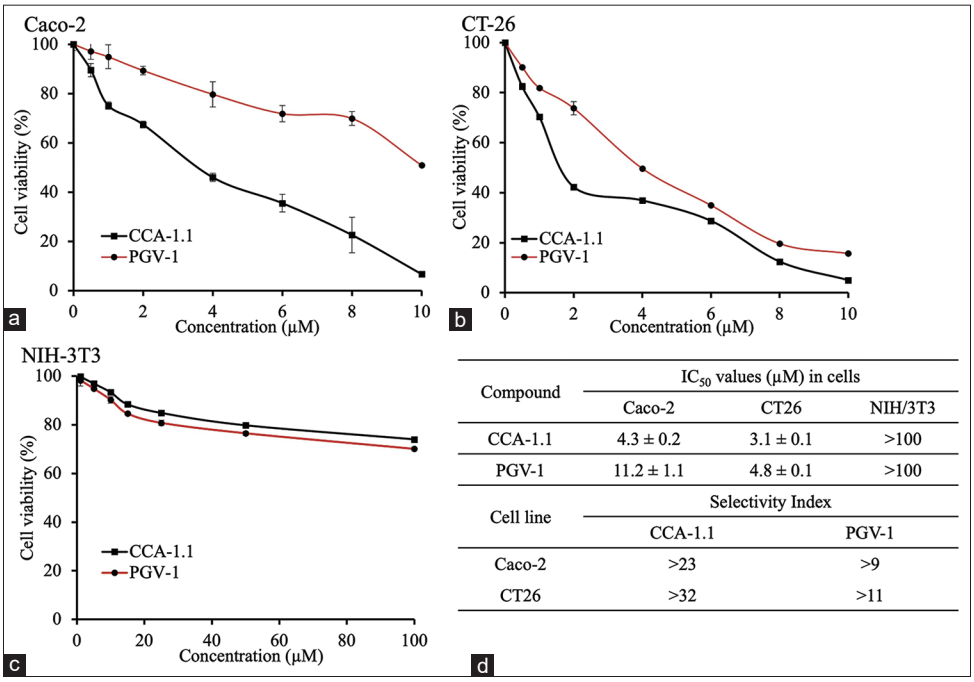
CCA-1.1 drastically ($P < 0.0001$) increased the G2/M population in Caco-2 cells. We also found that PGV-1 significantly ($P < 0.0001$) induced G2/M arrest with a 72.6% ±0.2% of the cell population [Figure 4a]. Similarly, in CT26 cells, both compounds significantly ($P < 0.0001$; $P < 0.01$) halted cell cycling progression at the G2/M phase [Figure 4b]. Interestingly, the subG1 population significantly ($P < 0.0001$) increased in both treated Caco-2 cells, but unchanged in CT26 cells. In apoptosis analysis, a similar percentage of $AnxV^+/PI+$ in treated Caco-2 cells was displayed [Figure 4c], while CCA-1.1 was more effective than PGV-1 in inducing apoptosis in CT26 cells [Figure 4d].

### CCA-1.1 and PGV-1 promote reactive oxygen species levels and cell senescence in colorectal cancer cells

Both CCA-1.1 and PGV-1 stimulated ROS generation in Caco-2 cells, with PGV-1 being the least strong [Figure 5a]. Doxorubicin served as a positive control. Similarly, in CT26 cells, CCA-1.1 showed the highest ROS production [Figure 5b]. Interestingly, treatment with CCA-1 did not stimulate ROS generation in NIH/3T3 non-cancerous cells, unlike PGV-1 and doxorubicin [Figure 5c]. In addition, CCA-1.1 and PGV-1 drastically ($P < 0.0001$) increased the senescent Caco-2 cells, with CCA-1.1 causing around 80% of cell senescence [Figure 5d and e]. Similar results were obtained in CT26 cells [Figure 5f and g]. Not surprisingly, doxorubicin-treated fibroblasts showed the highest percentage, but neither CCA-1.1 nor PGV-1 was found to induce senescence in NIH/3T3 cells [Figure 5h and i]. These data indicated that CCA-1.1-induced senescence, possibly through ROS induction with greater effect than PGV-1, while demonstrated milder effect in normal cells.

## DISCUSSION

This study is the first to evaluate the anti-tumorigenic ability of two curcumin analogs, CCA-1.1 and PGV-1, in a colorectal cancer animal model. PGV-1 or CCA-1.1 prevents the early stages of DMH-induced colorectal carcinogenesis, supported by their *in vitro* anticancer activities, including the selective induction of apoptosis, targeted cell cycle progression, and the inhibition of related cancer marker proteins.[2,10] Early preneoplastic hyperproliferative lesions generate aberrant crypt foci as intermediary indications of carcinogenesis in DMH-induced colorectal cancer models.[14-16] According to our findings, DMH-induced carcinogenesis in rats might be suppressed or prevented by these curcumin analogs




| Compound | $IC_{50}$ values (μM) in cells | | |
|---|---|---|---|
| | Caco-2 | CT26 | NIH/3T3 |
| CCA-1.1 | 4.3 ± 0.2 | 3.1 ± 0.1 | >100 |
| PGV-1 | 11.2 ± 1.1 | 4.8 ± 0.1 | >100 |
| Cell line | Selectivity Index | | |
| | CCA-1.1 | PGV-1 | |
| Caco-2 | >23 | >9 | |
| CT26 | >32 | >11 | |

**Figure 3:** Chemoprevention Curcumin Analog-1.1 (CCA-1.1) or Pentagamavunone-1 (PGV-1) inhibit colorectal cancer cell growth *in vitro*. The cell viability after treatment with PGV-1 or CCA-1.1 against (a) Caco-2, (b) CT26, and (c) NIH/3T3 cells. (d) $IC_{50}$ values of PGV-1 and CCA-1.1 and the selectivity index calculation. The graph represents average ± SD ($n = 3$)

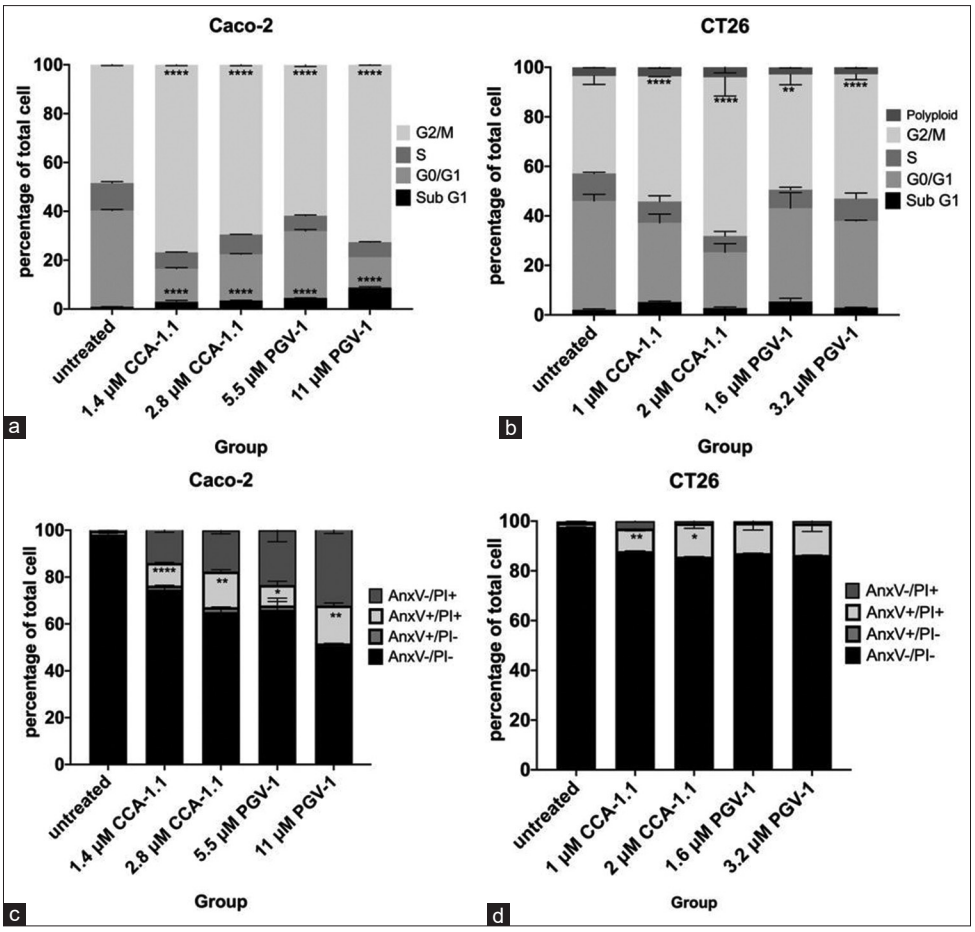



**Figure 4:** Chemoprevention Curcumin Analog-1.1 (CCA-1.1) or Pentagamavunone-1 (PGV-1) induce G2/M arrest and apoptosis in Caco-2 and CT26 cells. The distribution of Caco-2 (a) and CT26 (b) cells in each cell cycle phase. The distribution of cell population based on apoptosis staining in Caco-2 (c) and (d) CT26 (d) cells. Data are shown as the mean ± SD ($n$ = 3). *$P$<0.05; **$P$<0.01; ****$P$<0.0001 compared to the DMH-induced group

without noticeable impact on the hematological profile. A related study also confirm the stronger effect of PGV-1 than curcumin in preventing tumor formation in K-562 and 4T1-implanted mice.[2,3] Another curcumin analog, namely hexagamavunone-0, exhibits better chemopreventive activity than curcumin in reducing the number of nodules and COX-2 expression in DMH-induced rats.[12] Amidst the obstacles curcumin faces regarding its bioavailability, the option to modify the chemical structure could add some benefits to not only improve bioavailability but also obtain better safety and efficacy to eliminate tumors.

To better understand the cellular mechanism, *in vitro* tests using two different colorectal cancer cell lines were performed. In cell cycle progression, both compounds are revealed to induce mitotic catastrophe before entering the apoptosis stage. Prior studies report that CCA-1.1, as well as PGV-1, prevent mitosis progression in cancer cells.[2,8] Previous bioinformatic studies propose that CCA-1.1 or PGV-1 may target major mitotic kinases that possibly mediate their activity to induce cell cycle arrest.[7,17,18] Since senescence can also be triggered during G2/M arrest,[19] it is plausible that even with a high G2/M cell population, both PGV-1 and CCA-1.1 can provoke senescence of the cells. Senescence can also present as the cellular response in cancer cells. Many cancer chemotherapies induce premature senescence by promoting mitogenic pathway activation which is mediated by oxidative stress, then leading to steady cell cycle arrest.[19] Cellular senescence becomes one of the manifestations from excessive ROS.[20] This study shows that β-galactosidase activity was unaltered in NIH/3T3 cells, suggesting that the senescence induction by these curcumin analogs is selective in cancer cells.

Since these compounds are administered orally, our results also highlight the unique advantage of developing these analogs for anti-colorectal cancer therapies. However, due to their poor intestinal stability, a number of standard chemotherapy medicines are not often ingested orally.[21] Further investigation of these curcumin analogs using another treatment design may help establish a detailed mechanism of action for these compounds in cancer cells. This presents an attractive possibility for an oral pharmaceutical treatment for colorectal cancer.

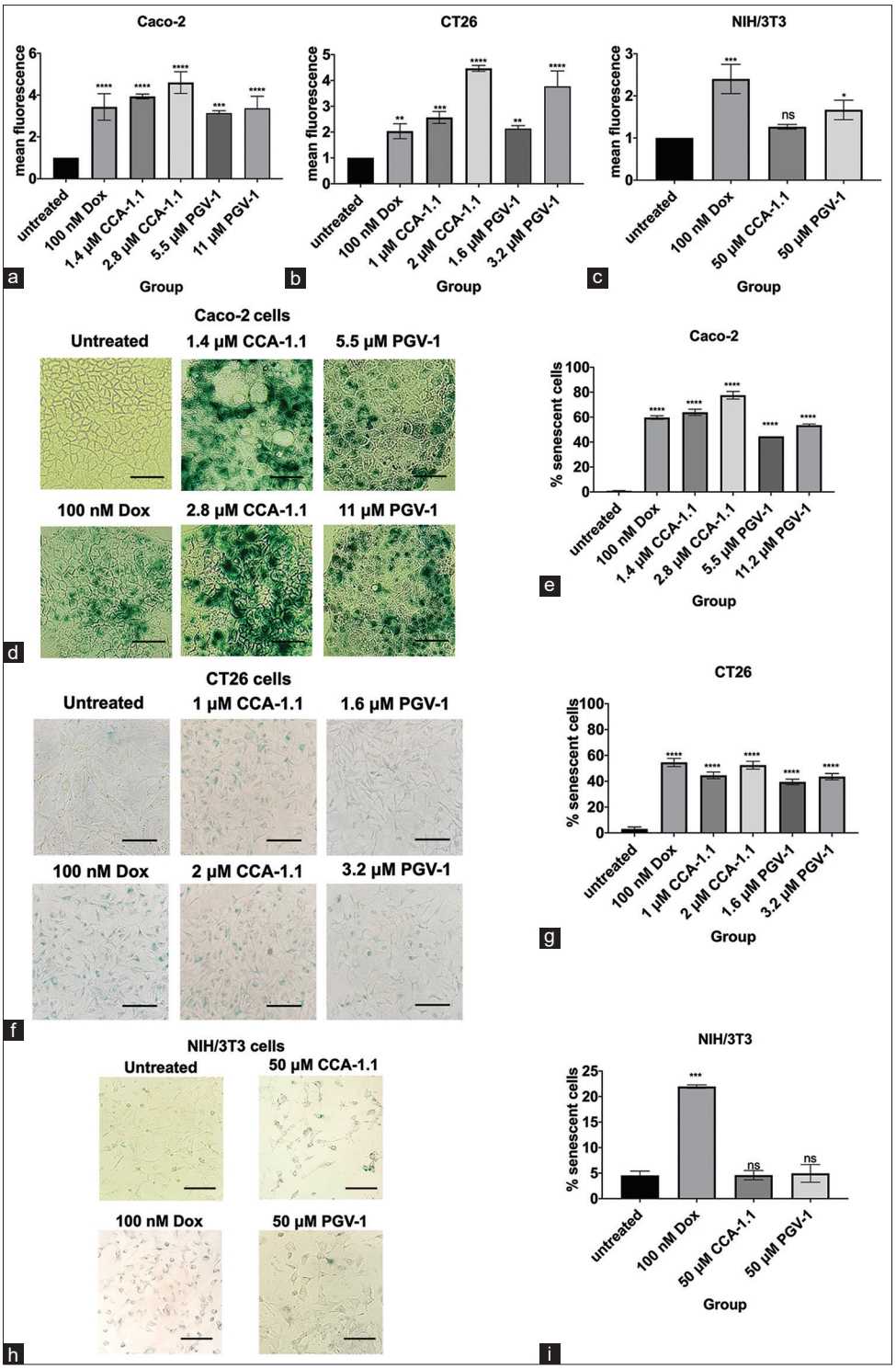



**Figure 5:** Chemoprevention Curcumin Analog-1.1 (CCA-1.1) or Pentagamavunone-1 (PGV-1) enhances reactive oxygen species (ROS) production and senescence. (a-c) ROS production. The mean fluorescence after treatment in (a) Caco-2, (b) CT26, and (c) NIH/3T3 cells are calculated against the untreated. (d-i) Cellular senescence. (d) The morphology and (e) quantification of senescent Caco-2 (d and e), CT26 (f and g), and NIH/3T3 (h and i) cells. Scale bar = 200 µm. Data are presented as mean ± SD ($n$ = 3). ns: not significant; *$P$<0.05; **$P$<0.01; ***$P$<0.001; ****$P$<0.0001 compared to the DMH-induced group

## CONCLUSION

Taken together, oral administration of CCA-1.1 and PGV-1 significantly prevents colorectal carcinogenesis and inhibits tumor formation compared to the DMH-induced group without affecting body weight and hematological parameters. Furthermore, CCA-1.1 and PGV-1 exhibit strong chemopreventive effects against colorectal cancer

at similar physiological activities in inhibiting cancer cell proliferation through cell cycle inhibition and senescence induction that led to cancer cell death.

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## Conflicts of interest

There are no conflicts of interest.

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