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ENZYCHEM LIFESCIENCES

KRIBB Korea Research Institute of Bioscience & Biotechnology

The synergistic effect of PLAG on the anti-tumor efficacy of AC-regimen via alleviating neutrophil tumor infiltration on breast tumor xenograft model

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ABSTRACT

Background: Tumor microenvironments (TME) provides promotion of tumor growth and induction of metastasis. The tumor-infiltrating neutrophil (TIN) is known as frequently observed in TME, which also contributes tumor promotion of abnormal-growth and metastasis. Effective destruction of TIN in TME might enhance therapeutic efficacy of chemotherapy for tumor reduction. In this experiment, we investigated to enhance therapeutic efficacy of PLAG using simultaneous treatment of PLAG and AC-regimen in the MDA-MB-231 breast cancer Xenograft model via modulating of TIN.

Methods: To investigate the synergistic effect on therapeutic efficacy of tumor using simultaneous treatment of PLAG and AC-regimen, we used MDA-MB-231 breast cancer Xenograft model. Tumor growth was evaluated in the AC-regimen alone and PLAG co-treated animals. AC-regimen was delivered via IP injection twice a week with dose of 2/20 and 5/50 mpk (Doxorubicin/Cyclophosphamide), and PLAG was daily administrated with 100 and 250 mpk. Tumor growth was calculated with 3day intervals. Neutrophil chemotaxis related chemokines, CXCL1/2/8 and circulating neutrophils were also evaluated with 2 weeks interval. Expression of apoptosis-related molecular markers, Bax/Bak and TIN in the tumor lesion was analyzed by immunohistochemistry (IHC).

Results: PLAG has synergistic effects decreasing the tumor burden in the PLAG and AC-regiment co-treated in Xenograft mice. In AC-regimen with 2/20 or 5/50 mpk treated groups, retardation of tumor growth was observed by calculating tumor size and processed apoptosis was proved by TUNEL and apoptosis-related proteins expression in the regressed tumor burden with AC-regimen. Modulated chemokine expression from tumor burden and subsequent neutrophil recruitment were also detected with dependent on tumor mass. In the PLAG co-treated group, smaller tumor burden than that in AC-regimen alone was consistently observed till sacrifice. It was confirmed that the tumor burden of the PLAG co-treated group with 5/50 AC-regimen was significantly decreased in a concentration-dependent manner compared to the AC-regimen alone group ($p<0.05$). Especially, in 250 mpk PLAG co-treated group, no tumor tissues were found in test mice on the sacrifice day. Significantly reduced chemokine expression and TIN in the PLAG added group were proved through IHC and chemokine analysis. Additionally, interruption of tumor growth, and reduced chemokine expression and TIN were observed in the PLAG alone treated groups. As summarized, PLAG has a prominent synergistic effect on regression of tumor burden in the co-treated with AC-regimen. Analysis of tumor tissue with IHC revealed that TIN was dramatically reduced in the PLAG co-treated group.

Conclusion: Taken together, PLAG has synergistic effects on decreasing the tumor burden through devastating tumor microenvironment by controlling of TIN upon incorporation with tumor destructive apoptosis by AC-regiment. Both apoptosis of tumor cell induced by AC-regimen and regulation of TIN by PLAG treatment will guarantee an improved elimination of malignant tumor burden.

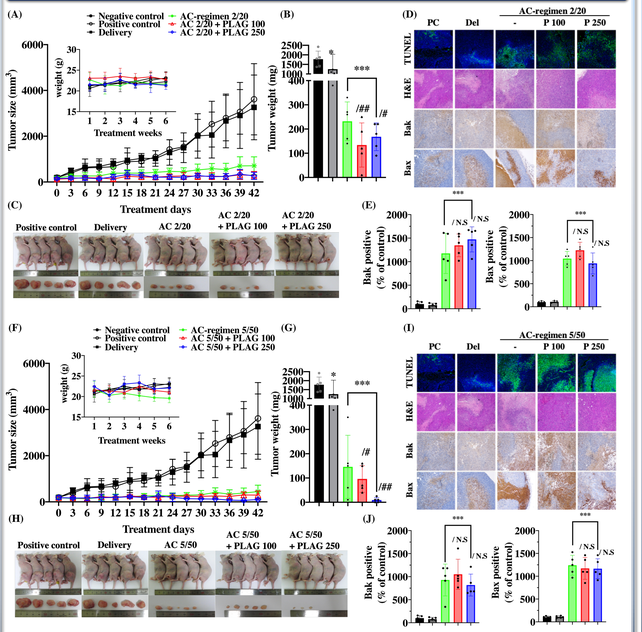
EXPERIMENTAL DESIGN

Drug	Dose (Total concentration)	Delivery vehicle	Delivery Method
PLAG	100 mpk/day, 250 mpk/day	Sterile PBS	Oral Administration (OA)/day, Everyday
Doxorubicin	24 mg/kg		Intraperitoneal (IP), 2 mg/kg/day, 2 injection/week
	60 mg/kg		Intraperitoneal (IP), 5 mg/kg/day, 2 injection/week
Cyclophosphamide	240 mg/kg		Intraperitoneal (IP), 20 mg/kg/day, 2 injection/week
	600 mg/kg		Intraperitoneal (IP), 50 mg/kg/day, 2 injection/week

AC-regimen delivery : every Tue/Fri 17:30

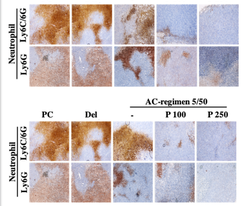
RESULT

Synergistic effect of PLAG with the AC-chemo regimen

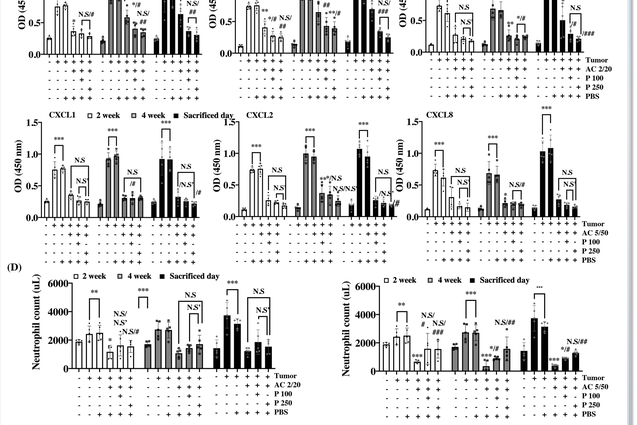


(**A**,**F**) The change of weekly recorded tumor mass in Xenograft mouse during AC-regimen treatment and PLAG co – treatment. (**B**,**G**) Analysis of Tumor weight of each group on sacrifice day. (**C**,**H**) Tumor mass in xenograft mice during AC-regimen and PLAG co-treatment. (**D**,**I**) H&E staining and apoptosis-related protein expression of tumor tissue in Xenograft mouse at sacrifice day. (**E**,**J**) The changes in apoptosis-related protein expression level in tumor tissue at sacrifice day. The positive area compared to the total tissue area was quantified by image J.
; Compared to positive control: *P<0.05, ***P<0.001 (each experiment n=5). Compared to regimen treated group: #P<0.05, ##P<0.01 (each experiment n=5). N.S., not significant.

Inhibitory effect of neutrophil chemotaxis

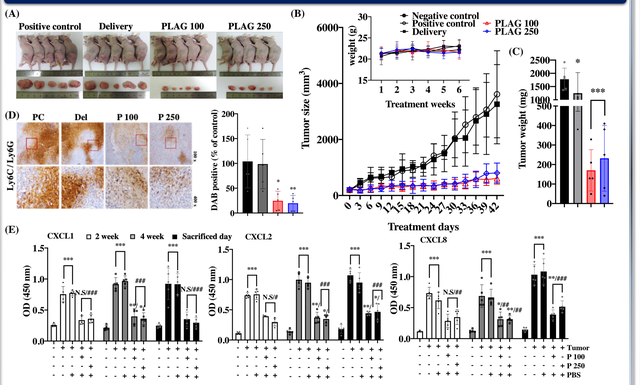


(**A**) Verification of tumor infiltration neutrophil using immunohistochemistry with antibodies. Anti-Ly6C+/Ly6G+ antibody and anti-Ly6G+ only antibody were used.
(**B**) Evaluation of tumor infiltrating neutrophil. Compared to negative control: *P<0.05, **P<0.01, ***P<0.001 (each experiment n=5). N.S., not significant. Compared to AC-regimen group: #P<0.05, ##P<0.01, ###P<0.001 (each experiment n=5). N.S.', not significant.

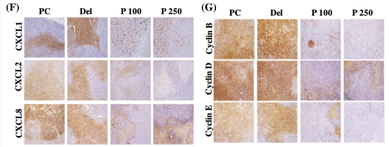


(**C**) The evaluation of secreting chemokines associated with neutrophil chemo-taxis in blood: *P<0.05, **P<0.01, ***P<0.001 (each experiment n=5). N.S., not significant. Compared to AC-regimen control group: #P<0.05, ##P<0.01, ###P<0.001 (each experiment n=5). N.S.', not significant. (**D**) The evaluation of neutrophil in blood: *P<0.05, **P<0.01, ***P<0.001 (each experiment n=5). N.S., not significant. Compared to AC-regimen control group: #P<0.05, ##P<0.01, ###P<0.001 (each experiment n=5). N.S.', not significant.

Growth inhibition of cancer by PLAG treatment

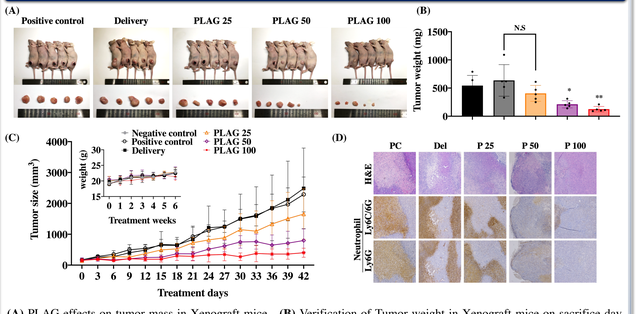


(**A**) PLAG effects on tumor mass in Xenograft mice (**B**) Verification of Tumor weight in Xenograft mice on sacrifice day. Compared to positive control: *P<0.05, ***P<0.001 (each experiment n=5). (**C**) Change of weekly counted tumor mass in PLAG treated Xenograft mice. (**D**) Verification of infiltration neutrophil using immunohistochemistry. (**E**) The evaluation of secreting chemokines associated with neutrophil chemo-taxis in blood: compared to negative control; *P<0.05, **P<0.01, ***P<0.001 (each experiment n=5). N.S., not significant. Compared to positive control; #P<0.05, ##P<0.01, ###P<0.001 (each experiment n=5). N.S.', not significant.



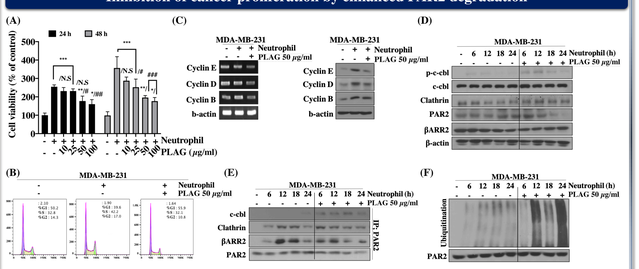
(**F**) Verification of neutrophil chemotaxis related chemokine expression in tumor using immunohistochemistry. (**G**) Verification of tumor cell cycle inducing factor expression in tumor using immunohistochemistry.

Inhibition of cancer growth and neutrophil infiltration by PLAG treatment



(**A**) PLAG effects on tumor mass in Xenograft mice (**B**) Verification of Tumor weight in Xenograft mice on sacrifice day. Compared to positive control: *P<0.05, **P<0.01 (each experiment n=5). N.S., not significant. (**C**) Change of weekly counted tumor mass in PLAG treated Xenograft mice. (**D**) Verification of tumor infiltration neutrophil using immunohistochemistry.

Inhibition of cancer proliferation by enhanced PAR2 degradation



(**A**) Inhibition of cell growth by PLAG treatment with dose-dependent manner in neutrophil-activated MDA-MB-231 breast cancer cells. Compared to negative control each week: *P<0.05, **P<0.01, ***P<0.001 (each experiment n=5). N.S., not significant. Compared to neutrophil stimulated only group each week: #P<0.05, ##P<0.01, ###P<0.001 (each experiment n=5). N.S.', not significant. (**B**) Inhibitory effect of PLAG on cell cycle activity in the neutrophil activated cancer cells. (**C**) Expression level of cell cycle related gene and protein was evaluated by PCR and Western blotting in the neutrophil and PLAG treated cells. (**D**) Verification of protein expression and phosphorylation related with PAR2 degradation in the PLAG and neutrophil co-treated activated cancer cells by western blot analysis. (**E**) Identification of PAR2 binding proteins using immunoprecipitation assay. (**F**) Degradation of PAR2 was verified with ubiquitin activity in the PLAG and neutrophil co-treated cancer cells by ubiquitination assay with anti-PAR2 antibody.

CONCLUSION

- **PLAG has synergistic effects on decrease of tumor burden through devastating tumor microenvironment by controlling of TIN (Tumor-infiltrating neutrophil).**
- **The treatment of PLAG effectively regulates the expression of neutrophil chemotactic chemokine in cancer tissues to control excessive infiltration of neutrophils.**
- **In addition, PLAG alone can regulate excessive growth by inhibiting the cell cycle of cancer tissues.**
- **The effect of PLAG inhibits cell cycle and chemokine expression by inducing PAR2 degradation in cancer tissues.**