1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol ameliorates chemoradiation-induced oral mucositis in a mouse model by regulating necroptosis

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ABSTRACT

Background: Currently, no approved drug exists to treat oral mucositis (OM) in patients receiving chemoradiation for the treatment of head and neck cancer. To address this limitation, we examined the therapeutic effect of 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG), which is an immune resolution accelerator that can effectively modulate the neutrophil recruitment against chemoradiation-induced oral mucositis (CRIOM) in a murine model. Additionally, we investigated whether necroptosis is involved in the pathogenesis of CRIOM in a murine model and the therapeutic effect of PLAG against the disease.

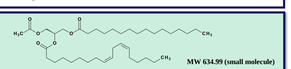
Methods: To investigate the improved effect of PLAG on CRIOM, we established the CRIOM-bearing BALB/c mouse model using concurrent treatments of 5-fluorouracil (100 mg/kg, i.p.) and X-radiation (20 Gy) on the heads and necks of mice. Phosphate buffer saline (PBS) or PLAG (100 and 250 mg/kg, p.o.) was administered daily. The body weights of mice were observed daily and on Day 9, the mice were sacrificed for extracting their tongues for further tissue analyses. The histopathological grading of OM was analyzed in the collected tongues. Human keratinocytes (HaCaT) were utilized to identify the mechanism of PLAG on regulating the necroptosis signal.

Results: On Day 9, chemoradiotherapy-treated (ChemoRT) mice had tongue ulcerations and experienced significant weight loss (Day 0: 26.18 ± 1.41 g; Day 9: 19.44 ± 3.26 g). They also had elevated serum macrophage inhibitory protein 2 (MIP-2) (control: 5.57 ± 3.49 pg/ml; ChemoRT: 130.14 ± 114.54 pg/ml) and interleukin (IL)-6 (control: 198.25 ± 16.91 pg/ml; ChemoRT: 467.25 ± 108.12 pg/ml) levels. ChemoRT-treated mice who received PLAG exhibited no weight loss (Day 0: 25.78 ± 1.04 g; Day 9: 26.46 ± 1.68g) and had lower serum MIP-2 (4.42 ± 4.04 pg/ ml) and IL-6 (205.75 ± 30.41 pg/ml) levels than ChemoRT-treated mice without the PLAG treatment. Tongue tissues of mice who received PLAG also displayed lower phosphorylation levels of necroptotic signaling proteins (RIPK1/3 and MLKL). Chemoradiation induced the release of damaged-associated molecular patterns (DAMPs), which activates the necroptosis signaling pathway if not promptly eliminated intracellularly in HaCaT cells. PLAG promoted the effective removal of DAMPs, thereby downregulating the activation of the necroptosis signaling pathway.

Conclusion: On day 9, the tongues of micetreated with chemoradiotherapy activated the necroptosis signaling pathway. As a result, cells from oral mucosa released DAMPs and pro-inflammatory cytokines to induce neutrophil migration to the inflamed oral epithelium. PLAG ameliorated the degree of OM by terminating the necroptosis signaling pathway. These data suggest that PLAG may be a useful therapeutic agent for the treatment of CRIOM.

PLAG

(**EC-18**, 1-Palmitoyl-2-Linoleoyl-3-Acetyl-rac-Glycerol)

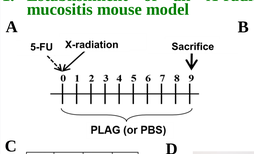


MW 634.99 (small molecule)

- **The Therapeutic Effect of PLAG against Oral Mucositis in Hamster and Mouse Model.** ***Front Oncol.*** **2016; 6: 209**
- **1-Palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol ameliorates chemoradiation-induced oral mucositis.** ***Oral Dis.*** **2020 Jan;26(1):111-121**

RESULTS

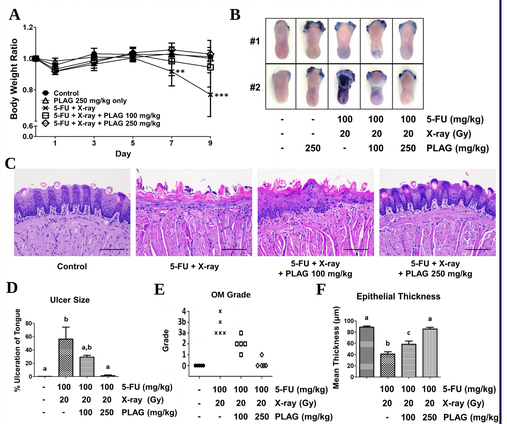
1. Establishment of an X-radiation and 5-FU–induced oral mucositis mouse model



(A) On Day 0, the mice were divided into different groups. The mice then received 100 mg/kg 5-FU intraperitoneally and 20 Gy X-radiation to the head and neck region. PBS or PLAG was administered orally each day until Day 9. (B) Changes in body weight were recorded each day and compared between groups. Data are shown as mean ± SEM (#p < .05, ##p < .001, ###p < .001 vs. Day 0). (C) Mice were sacrificed on Day 9, and their harvested tongues were stained with toluidine blue. (D) Tongues from each treatment group were stained with H&E. Scale bar = 201 μm. (E) Histopathologic grading was determined for each treatment group.

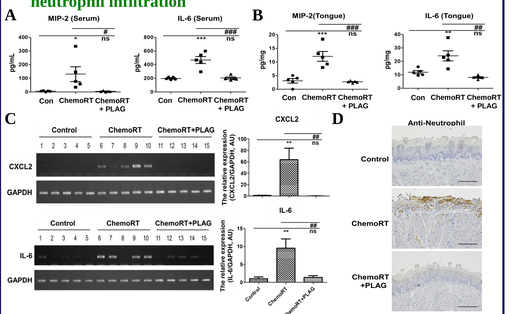
In chemoradiation-induced oral mucositis mice model

2. PLAG attenuated chemoradiation-induced oral mucositis



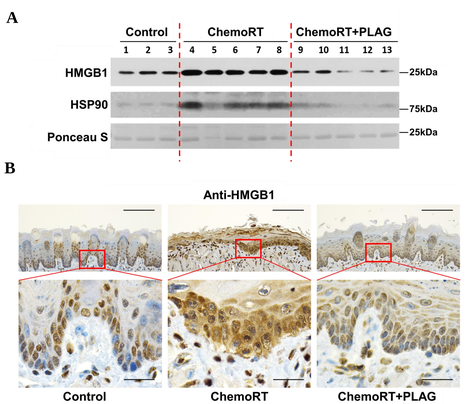
(A) ChemoRT (100 mg/kg 5-FU and 20 Gy X-radiation) was administered to the mice, with or without the addition of 100 mg/kg or 250 mg/kg PLAG. Body weight was recorded daily. Data are shown as mean ± SEM (p < .01, ***p < .001 vs. Day 0). (B) On Day 9, mice were sacrificed, and the harvested tongues were stained with toluidine blue. (C) Tongues from each treatment group were stained with H&E. (D) Ulcer size was measured using ImageJ, and the ratio of ulcer area/total area was expressed as a percentage. (E) Histopathologic grading was determined for each treatment group. Scale bar = 201 μm. (F) Oral mucosa epithelial thickness was measured at 20 randomly selected sites in tissue slides and compared between groups. Data represent mean ± SEM. Significant differences between groups with p < 0.05 are marked with different letters (D and F).**

3. PLAG ameliorated proinflammatory cytokine release and neutrophil infiltration



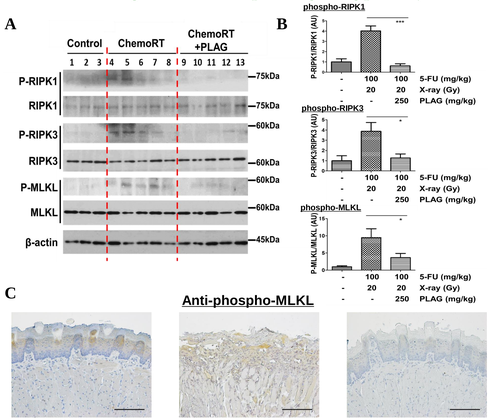
(A) Samples obtained from control, ChemoRT (100 mg/kg 5-FU + 20 Gy) and ChemoRT + PLAG 250 mg/kg group mice on Day 9 were used to detect serum levels of the proinflammatory cytokines MIP-2 and IL-6. (B) Tongue extracts were used to detect MIP-2 and IL-6 levels. (C) Expression of MIP-2 (CXCL2) in tongue tissues was examined at the transcriptional level using RT-PCR. Relative expression was compared between groups. (D) IL-6 mRNA expression was detected using RT-PCR, and relative expression was compared between groups. (E) Immunohistochemistry was performed with the neutrophil-specific antibody NIMP-R14. The ChemoRT group displayed neutrophil infiltration in the epithelium, whereas the PLAG co-treated group did not exhibit this infiltration. Neutrophils are stained brown. Scale bar = 201 μm. Data are shown as mean ± SEM (*/#p < .05, **/##p < .01, */###p < .001)**

4. PLAG reduced the release of DAMPs



(A) Levels of DAMPs in the serum from control, ChemoRT (100 mg/kg 5-FU + 20 Gy) and ChemoRT + PLAG 250 mg/kg group mice were examined by Western blotting. HMGB1 and Hsp90 were detected in the serum samples obtained on Day 9. Ponceau S staining of membrane proteins was used to demonstrate comparable protein loading. (B) HMGB1 localization was observed by immunohistochemistry. Cytoplasmic HMGB1 was positively stained in the ChemoRT group. Nuclei are stained blue; HMGB1 is stained brown. Scale bars = 201 μm (upper panels) and 40.1 μm (lower panels).

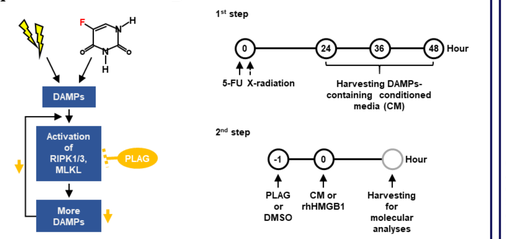
5. PLAG downregulated the necroptosis signaling pathway



(A) Protein levels of the necroptosis markers RIPK1, RIPK3 and MLKL were detected by Western blotting in tongue lysates from control, ChemoRT (100 mg/kg 5-FU + 20 Gy) and ChemoRT + PLAG 250 mg/kg groups. (B) Band densities of phosphorylated RIPK1 (P-RIPK1), RIPK3 (P-RIPK3) and MLKL (P-MLKL) were compared to band densities of total RIPK1, RIPK3 and MLKL using ImageJ. (C) P-MLKL was visualized by immunohistochemistry. P-MLKL is stained brown. Scale bar = 201 μm. Data are shown as mean ± SEM (*p < .05, ***p < .001 vs. ChemoRT using Student's t-test)

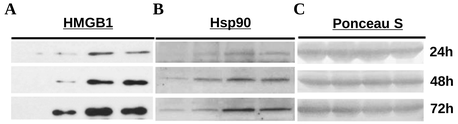
In Vitro study

1. Experimental schematic design for the study of the effect of DAMPs released from injured cells by chemoradiation



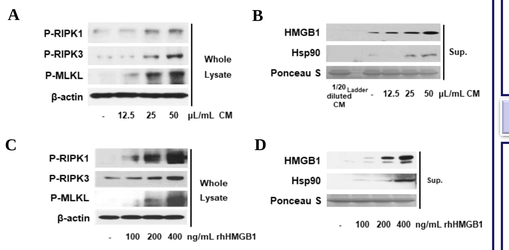
(A) Epithelial cells treated with ChemoRT release DAMPs. These DAMPs activate RIPK1/3 and MLKL in nearby cells. The necroptotic pathway leads to additional DAMP release, creating a positive feedback loop and amplifying necroptosis signals. PLAG mitigates these necroptosis signals, leading to less DAMP release, mitigating the positive feedback loop. (B) HaCaT cells were treated with ChemoRT, and released DAMP-containing conditional media (CM) were collected. Next, cells were pre-treated with PLAG for 1 hour before CM or rhHMGB1 was added. The cells and culture media were then harvested for further molecular analyses.

2. Released DAMPs in ChemoRT-treated HaCaT cells



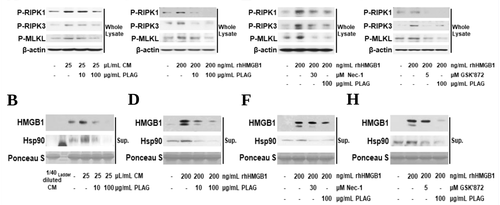
HaCaT cells treated with 5-FU and/or X-radiation released (A) HMGB1 and (B) Hsp90 at 24, 36, and 48 hours of treatments. (C) Ponceau S staining was performed to demonstrate comparable protein loading.

3. Released DAMP-containing CM and rhHMGB1 induce necroptosis and subsequent DAMP release



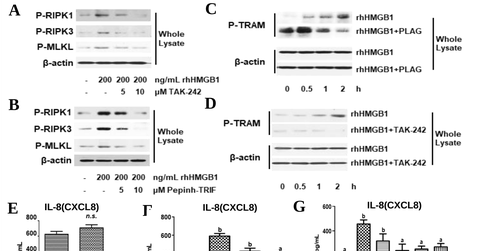
(A) Necroptosis signaling was detected in cells treated with CM after 2 hours. (B) Subsequent release of HMGB1 and Hsp90 were detected in culture supernatant after 72 hours of CM treatment. To demonstrate the amount of DAMPs in CM added to HaCaT cells, 50 μL of CM from ChemoRT-treated cells was diluted to 1/20 in DMEM culture media. (C) Necroptosis signaling activity was detected in cells treated with rhHMGB1 after 2 hours. (D) Released HMGB1 and Hsp90 were detected in culture supernatant after 72 hours of rhHMGB1 CM treatment.

4. PLAG mitigated necroptosis signaling and DAMP release.



(A) Necroptotic signals of cells treated with CM and PLAG were detected after 2 hours. (B) HMGB1 and Hsp90 were detected in culture supernatant harvested after 72 hours. To demonstrate the amount of DAMPs in CM added to HaCaT cells, 25 μL of CM from ChemoRT-treated cells was diluted to 1/40 in DMEM culture media. (C, E, and G) Necroptotic signals of cells treated with rhHMGB1 were detected after 2 hours. (D, F, and H) HMGB1 and Hsp90 were detected in culture supernatant harvested after 72 hours.

5. PLAG ameliorated necroptosis by regulating the TLR4 endosomal signaling cascade and modulates IL-8 release induced by CM and rhHMBG1



Phosphorylation of necroptotic signals detected in cells treated with (A) rhHMGB1 and TAK-242 and (B) rhHMGB1 and TRIF inhibitor. (C) P-TRAM was observed in 200 ng/mL rhHMGB1-only treated cells, and 200 ng/mL rhHMGB1 and 100 μg/mL PLAG-treated cells at 0, 0.5, 1, and 2 hours. P-TRAM was also observed in 200 ng/mL rhHMGB1-only treated cells, and 200 ng/mL rhHMGB1 and 10 μM TAK-242-treated cells at 0, 0.5, 1, and 2 hours. IL-8 was detected in (E) CM, (F) cells treated with CM, and (G) cells treated with rhHMGB1. Data represent mean ± SEM. Significant differences between groups (p<0.05) are marked with different letters (F and G). n.s.=not significant.

CONCLUSION