Suppression of tumor progression by thioredoxin-interacting protein-dependent adenosine 2B receptor degradation in a PLAG-treated Lewis lung carcinoma-1 model of non–small cell lung cancer

Abstract

Extracellular adenosine in the tumor microenvironment plays a vital role in cancer development. Specifically, activation of adenosine receptors affects tumor cell growth and adenosine release.

We examined the anti-tumor efficacy of 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG) in animal models, revealing the role of PLAG in inhibiting tumor progression by promoting the degradation of adenosine 2B receptors (A2BRs) in tumors. PLAG induced the expression of thioredoxin-interacting protein (TXNIP), a type of arrestin that accelerates A2BR internalization by interacting with A2BR complexes containing β-arrestin. Engulfed receptors bound to TXNIP were rapidly degraded after E3 ligase recruitment and ubiquitination, resulting in early termination of intracellular signals that promote tumor overgrowth. However, in control cancer cells, A2BRs bound to protein phosphatase 2A and were returned to the cell membrane instead of being degraded, resulting in continuous receptor-mediated signaling by pathways including the Raf-Erk axis, which promotes tumor proliferation. A TXNIP-silenced cell-implanted mouse model and TXNIP knockout (KO) mice were used to verify that PLAG-mediated suppression of tumor progression is dependent on TXNIP expression. Increased tumor growth was observed in TXNIP-silenced cell-implanted mice, and the anti-tumor effects of PLAG, including delayed tumor overgrowth, were greatly reduced. However, the anti-tumor effects of PLAG were observed in cancer cell-implanted TXNIP-KO mice, which indicates that PLAG produces anti-tumor effects by enhancing TXNIP expression in tumor cells.

These essential functions of PLAG, including delaying tumor growth via A2BR degradation, suggest innovative directions for anticancer drug development.

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Keywords: 1-Palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG), Thioredoxin-interacting protein, Adenosine 2B receptors, Degradation, Signal termination

Abbreviation: PLAG, 1-Palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol; A2BR, Adenosine 2B Receptor; TXNIP, Thioredoxin interacting protein; NEDD-4, Neural precursor cell-expressed developmentally downregulated protein 4; PP2A, Phosphatase 2A; KO, Knock-out; eAdo, extracellular Adenosine;GPCR, G-protein coupled receptors; MAPK, Mitogen-activated protein kinase; LLC-1, Lewis Lung Carcinoma; MOA, Mechanism of Action; TME, Tumor microenvironment; EGFR, Epidermal growth factor receptor; RTK, Receptor tyrosine kinase.

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Introduction

Extracellular adenosine, which is mainly secreted by tumor tissue, stimulates tumor cell proliferation and regulates the immune response, which is advantageous for tumor development [1–3]. In particular, the cell signaling pathways activated through the adenosine receptor family induce both overactivation of cancer cells and inhibition of the anticancer immune response. A recent study found that extracellular adenosine stimulates adenosine 2B receptor (A2BRR), which induces tumor growth, promotes metastasis, and effectively induces the tumor microenvironment (TME). Induction of A2BR activity by extracellular adenosine activates tumor growth–related signaling pathways, such as the Erk pathway, and induces cAMP accumulation. In addition, antigen-presenting cells, which express A2BRRs, in the TME promote tumor hyperactivity through inhibition of cytotoxic T-cell activity [4–9]. In previous studies, the specific inhibition of A2BRRs effectively inhibited tumor overgrowth in multiple tumor models [10–12]. Therefore, the inhibition of A2BRRs in tumors has become an important topic of cancer research.

A2BR is a type of G protein-coupled receptor (GPCR) that is activated when it binds to extracellular adenosine. Ligand-binding receptors initiate intracellular trafficking, and endocytosis of GPCRs occurs during the receptor desensitization process [13–15]. Recent studies have shown that intracellular signaling by ligand-specific receptors is induced during intracellular trafficking [16–18]. Endocytosis and intracellular GPCR signaling rely primarily on assembled complexes, such as those containing arrestins. GPCRs in endosomes activate signaling pathways, such as the mitogen-activated protein kinase (MAPK) pathway and contribute to tumor cell proliferation [19–21]. Whether an internalized GPCR is degraded or returns to the cell membrane is determined by the assembly of a receptor–protein complex [22–25].

Thioredoxin-interacting protein (TXNIP), a member of the α-arrestin family, is an important scaffold protein that induces endocytosis of receptors and transport proteins [26,27]. α-Arrestin, which contains PX/YW motifs, recruits E3 ligases and induces receptor ubiquitination and degradation to quickly and effectively terminate intracellular signaling [28–30]. Rapid adenosine receptor internalization and degradation are optimal processes for terminating intracellular signaling initiated by adenosine receptor activation, preventing excessive and continuous receptor activation.

1-Palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG) is present in the antlers of sika deer and can be synthesized from glycerol, palmitic acid, and linoleic acid. Previous studies have shown that PLAG can effectively alleviate symptoms of severe inflammatory diseases, such as acute lung injury, oral mucositis caused by chemoradiotherapy, and gout [31–34]. In addition, the abnormal metastasis of A549 non–small cell lung cancer cells and the excessive growth of the triple-negative breast cancer cell line MDA-MB-231 were also effectively inhibited by PLAG [35,36]. Based on these results, we investigated the inhibitory effect of PLAG on tumor progression in Lewis lung carcinoma (LLC1) cell-implanted mouse models. PLAG treatment effectively controlled tumor progression by inducing TXNIP expression. TXNIP is associated with the rapid internalization and degradation of A2BRRs, resulting in early termination of receptor-mediated signaling pathways that promote tumor proliferation, including the eCal/Erk axis.

Materials and methods

Test substance (PLAG) synthesis and production

PLAG was manufactured and provided by the New Drug Production Headquarters, a Good Manufacturing Practice facility of Enzymchem Lifesciences Corporation (Jecheon-si, South Korea), and was stored according to information provided by the manufacturer.

Cell culture

LLC1 cells were obtained from American Type Culture Collection (ATCC, MD, USA). Cells were grown in Dulbecco’s modified Eagle medium (WELGENE, Seoul, Korea) containing 10% fetal bovine serum (RMBio, Missoula, MO, USA) and 1% antibiotics (100 mg/mL streptomycin, 100 U/mL penicillin) at 37°C in a 5% CO2 atmosphere.

Immunofluorescence staining

LLC1 cells were seeded on a glass coverslip in a 12-well plate and incubated until they reached 60% confluence. The cultures were then treated with PLAG and adenosine at 37°C in a 5% CO2 atmosphere. The cells were then fixed with 3.7% formaldehyde for 20 min and permeabilized with 0.2% Triton X-100. To stain specific proteins, the cells were fixed with 3.7% formaldehyde for 20 min, washed twice with phosphate-buffered saline (PBS) with Tween 20 (PBST), and incubated with specific antibodies overnight at 4°C. The cells were then washed twice with PBST and incubated with secondary antibodies. Fluorescence was detected by confocal microscopy (Carl Zeiss, Thornwood, NY, USA). The amount of fluorescence was quantified using ImageJ software.

Analysis of protein assemblies by immunoprecipitation

LLC1 cells were treated with PLAG and stimulated with adenosine for various times at 37°C in a 5% CO2 atmosphere. The cells were then lysed in ice-cold immunoprecipitation lysis buffer (25 mM Tris-HCL, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5% glycerol). The extracted proteins were incubated with Surebeads Protein G-specific antibody-bound magnetic beads (Bio-Rad, CA, USA). The beads were then washed with PBST, and the target proteins were eluted in 1 x sample buffer and analyzed by western blotting.

Analysis of protein degradation by ubiquitination assay

LLC1 cells were treated with PLAG and stimulated with adenosine for various times after pre-treatment with 10 μM MG132 for 2 hours at 37°C in a 5% CO2 atmosphere. The cells were then lysed using ice-cold immunoprecipitation lysis buffer. The extracted proteins were incubated with Surebeads Protein G-specific antibody-bound magnetic beads (Bio-Rad), and the beads were then washed with PBST. Ubiquitinated A2BRR was eluted using a 1 x non-reducing sample buffer and analyzed by western blotting.

Gene silencing by small interfering RNA (siRNA) and small hairpin RNA (shRNA)

siRNA was purchased from Thermo Fisher Scientific (Waltham, MA, USA), and the shRNA plasmid was purchased from Origene Technologies (Rockville, MD, USA). For transient transfection, cells were washed twice with PBS. The target RNA was resuspended in Opti-MEM (Thermo Fisher Scientific) containing Lipofectamine 2000 (Thermo Fisher Scientific). The target RNA mixtures were placed in a cell plate for transfection. After transfection, the cells were incubated for 72 hours for target protein silencing. Then, silenced cells were selected by treatment with 10 μg/mL puromycin for 4 days, and shRNA-transfected cells were collected.

Lysosomal activity analysis

LLC1 cells were seeded in black 96-well plates, and intracellular lysosomal activity was detected using LysoSensor (Invitrogen, Carlsbad, CA, USA). Lysosomal fluorescence was quantitatively measured with a Varioskan LUX.
device (Thermo Fisher Scientific). The cells were then treated with PLAG, and LysoTracker was added using an injector. After the test dose of adenosine was administered, lysosomal fluorescence was measured at 5-min intervals. The experiment was performed at 37°C in a 5% CO₂ atmosphere.

Tumor implantation (syngeneic implantation)

Five-week-old male C57BL/6J mice were obtained from Daehan Bio-Link (Yong-in, South Korea) and housed in sterile filter-topped cages. The animals (n=6 for each treatment group) were anesthetized using isoflurane and placed in the right lateral decubitus position. A total of 1 × 10⁶ LLC1 cells in a solution containing 50 µL of culture medium and 50 µL of Matrigel (BD Biosciences, NJ, USA) were subcutaneously injected into the right-side thigh using a 29-G needle attached to a 0.5-mL insulin syringe (Becton Dickinson, NJ, USA). The mice were then allowed to rest on a heating pad until they fully recovered. Starting 3 days after cell implantation, PLAG was orally administered daily (n=6 mice per group). The positive control group (n=6 mice) was not treated. The tumor burden was calculated every 3 days after implantation. Tumor volume was approximated by a simplified ellipsoidal formula: (short axis) x (short axis) x (long axis)/2. Mice were euthanized when the longest axis of the tumor reached 2.2 cm. The animals were sacrificed 4 weeks after implantation and perfused with PBS. The tumors were extracted and fixed with 10% formaldehyde. Immunohistochemical (IHC) staining was performed on tissue sections to examine tissue morphology. All animal experiments were approved by the Institutional Animal Care and Use Committee of Korea Research Institute of Bioscience & Biotechnology (approval number: KIRIBB-AEC-20130).

TXNIP-KO mice study

TXNIP-KO mice were donated by Dr. In-Pyo Choi at the Immunotherapy Convergence Research Center, Korea Research Institute of Bioscience and Biotechnology. Implantation and analysis of LLC1 cells were performed as described above.

Overall survival analysis

Kaplan–Meier survival curves were plotted for the 60-day period after cancer cell implantation. Treatments were performed until the final observation week except in cases of death or moribundity.

Immunohistochemistry staining (IHC)

Tumors were fixed in 10% formaldehyde, embedded in paraffin, and sectioned into 5-µm slices. The sections were treated with 3% H₂O₂ for 10 min to block endogenous peroxidase activity and then blocked with bovine serum albumin. Then, the sections were washed with Tris-buffered saline with Tween and incubated with specific antibodies overnight at 4°C. Negative controls were incubated with normal serum IgG for the species from which the primary antibody was obtained. The 3,3’-diaminobenzidine-stained area was analyzed using ImageJ software.

Statistics

The data were analyzed using Prism 9 (GraphPad Software, La Jolla, CA, USA). A value of p<0.05 was considered significant.

Results

PLAG induces anti-tumor effects and improves survival through rapid A2BR degradation

The tumor burden was observed in syngeneic LLC1 cell-implanted mice, and the anti-tumor effect of PLAG was investigated according to the experimental schedule (Figure 1A). The tumor size increased markedly 2 weeks after implantation, and approximately 5 g of tumor tissue was observed 27 days after drug delivery. However, tumor growth was clearly delayed by PLAG treatment (Figure 1B, C). The tumor weight was decreased by 65% in 50 mg/kg PLAG-treated mice and by 86% in 100 mg/kg PLAG-treated mice (Figure 1D). Over 60 days, five of six mice died in the positive control group, and five of six mice survived in the group treated with 50 mg/kg PLAG (Figure 1E).

A2BR expression and cRaf/Erk phosphorylation were observed in tumor tissue. However, reduced A2BR expression and dephosphorylation of the cRaf/Erk axis were observed in mice treated with PLAG. High expression levels of TXNIP, which are closely related to biological activities, such as the promotion of A2BR degradation and the reduction of cRaf/Erk axis activity, were observed in PLAG-treated mice (Figure 1F–H).

PLAG promotes A2BR degradation by activating ubiquitination

The early termination of cRaf/Erk axis signaling and its effect on cell proliferation after A2BR degradation in PLAG-treated cells were verified using adenosine-treated LLC1 cells. Western blotting of adenosine-treated cells showed that cRaf/Erk signaling activity occurred for 6 hours in the presence of A2BRs, whereas A2BR degradation and cRaf/Erk inactivation were observed within 3 hours in PLAG co-treated cells (Figure 2A). Ubiquitination, which is a major cause of A2BR degradation, was observed within 3 hours in PLAG co-treated cells. Furthermore, PLAG treatment increased A2BR ubiquitination and lysosome activity (Figure 2B, D). Complexes containing A2BRs, β-arrestin-2, and phosphatase 2A (PP2A) were detected in adenosine-treated cells. When β-arrestin-2 and PP2A assemble, Rab4 is recruited, and this combination promotes receptor endocytosis and intracellular trafficking and facilitates the return of the receptor to the membrane. By contrast, in PLAG co-treated cells, A2BR bound to TXNIP, followed by binding to β-arrestin-1 and -2 and the E3 ligase neural precursor cell-expressed developmentally downregulated protein 4 (NEDD4) (Figure 2C, E, F and Supplementary Figure 2). The NEDD4–A2BR complex underwent ubiquitination and receptor degradation.

PLAG induces TXNIP expression and membrane localization

The TXNIP expression level in PLAG-treated cells was examined by western blotting, and TXNIP localization was examined by confocal microscopy. TXNIP showed increased expression in PLAG-treated cells and was localized to plasma membranes, suggesting that PLAG plays a major role in the rapid internalization and degradation of A2BR receptors (Figure 3).

Anti-tumor effects of PLAG during tumor progression

The anti-tumor effects of PLAG in LLC1 cell-implanted mice, including tumor size and A2BR-mediated signal modulation, were analyzed weekly. The size and weight of tumors did not increase in PLAG-treated mice during tumor development (Figure 4A). In particular, TXNIP expression decreased with tumor growth in mice but was maintained in PLAG-treated mice. cRaf/Erk phosphorylation, which is considered a major signal for tumor growth, was observed in untreated mice but not in PLAG-treated mice (Figure 4C–F). These results suggest that the A2BR/cRaf/Erk axis is directly
Suppression of tumor progression by thioredoxin-interacting protein-dependent G.T. Kim et al. Neoplasia Vol. 31, No. xxx 2022

Figure 1. Analysis of the effectiveness of PLAG on tumor development. A. Experimental design used to assess the effects of PLAG in an LLC1-implanted syngeneic mouse model. B. Tumor size reduction in PLAG-treated mice. C. The increase in tumor size in each group was estimated at 3-day intervals. D. The reduction in tumor weight was calculated in mice treated with PLAG for 4 weeks. E. Analysis of survival rates for PLAG-treated mice (survival/test mice). F. Protein expression and phosphorylation in tumor-induced tissues were analyzed using western blotting. G, H. Proteins associated with tumor progression were analyzed using immunohistochemical (IHC) staining. Compared to normal mice: ***p<0.001; Compared to the tumor only: #p<0.033, ###p<0.001 (each experiment n=6, n=4 for IHC analysis). N.S, Not significant. Mean±SD.

Figure 2. PLAG promotes degradation of A2BR through receptor ubiquitination. A. Alterations of A2BRs and signaling molecules in cells co-treated with adenosine and PLAG were tracked over time. B. Ubiquitination of A2BRs in cells co-treated with adenosine and PLAG. C. Changes in proteins associated with A2BR degradation in cells treated with adenosine alone or co-treated with PLAG. D. Lysosomal activity in PLAG-treated cells. E. Analysis of intracellular A2BR trafficking in cells co-treated with adenosine and PLAG. F. Recycling and co-localization of Rab4 and A2BRs in PLAG-treated cells (blue, nucleus; green, A2BR; red, Rab4).
linked to tumor overgrowth. Ubiquitination of A2BRs (i.e., degradation) was continuously observed in PLAG-treated mice and is suggested to play an essential role in reducing tumor progression.

**PLAG-mediated activities, such as A2BR degradation and anti-tumor signaling, are not observed in TXNIP-silenced cells**

The effectiveness of PLAG in TXNIP-silenced LLC1 cells was analyzed to verify the role of PLAG in inhibiting TXNIP-dependent tumor growth and the early termination of cRaf/Erk axis signaling by A2BR degradation. LLC1 cell growth was completely attenuated after adenosine treatment in PLAG co-treated cells, and this effect was absent in TXNIP-silenced cells (Supplementary Figure 4). Additionally, the A2BR degradation and cRaf/Erk dephosphorylation observed in PLAG-treated cells were not observed in TXNIP-silenced cells (Figure 5A). The ubiquitination and lysosomal activity involved in A2BR degradation in PLAG-treated cells were also absent in TXNIP-silenced cells (Figure 5B–D). TXNIP recruits A2BR complex proteins, such as β-arrestin-1/NEDD4, to degrade internalized receptors, but A2BRs bound to β-arrestin-2/PP2A in TXNIP-silenced cells return to the membrane, resulting in receptor recycling (Figure 5E–G).

**The anti-tumor effects of PLAG are dependent on the regulation of TXNIP expression in tumors**

The anticancer effect of PLAG was investigated in TXNIP-silenced cell-implanted mice. Compared with that in wild-type (WT) cell-implanted mice, a greater than 70% decrease in the inhibition of tumor progression was observed when PLAG was administered to TXNIP-silenced cell-implanted mice (Figure 6A). The 60-day survival rate was 100% in PLAG-treated mice implanted with WT cells. However, five of six mice implanted with TXNIP-silenced cells died, with no PLAG-mediated increase in survival rate.
(Figure 6B). TXNIP is a tumor suppressor protein [37,38], and TXNIP-silenced tumors may exhibit an aggressive phenotype. Additionally, PLAG did not reduce tumor size in TXNIP-silenced cell-implanted mice (Figure 6C). Modification of intracellular signaling-related proteins, such as A2BRs, and reduction of cRaf/Erk activity by PLAG treatment were not observed in TXNIP-silenced tumor-bearing mice (Figure 6D). Tumor tissue analysis of TXNIP-silenced tumor-bearing mice showed that they did not exhibit A2BR degradation or reduced cRaf/Erk phosphorylation (Figure 6E). PLAG had anticancer effects in TXNIP-KO mice implanted with WT tumors, whereas PLAG did not produce these effects in TXNIP-KO mice implanted with TXNIP-silenced tumors (Supplementary Figure 6). Therefore, PLAG-mediated induction of TXNIP in tumor tissue plays a pivotal role in the anticancer effects of PLAG.

**Discussion**

Recent reports have shown that adenosine plays an important role in the TME, resulting in tumor growth. In particular, activation of A2BRs by adenosine induces excessive tumor formation and creates a tumor-promoting microenvironment in which tumors can grow excessively by activating multiple signaling pathways, such as the MAPK pathway and Akt axis. Thus, targeting approaches to regulate A2BR activity is an important therapeutic area for anticancer research.

This study demonstrates the effect of inhibiting tumor overgrowth by inducing A2BR degradation through PLAG treatment. In the LLC1 cell-implanted mouse model, PLAG reduced tumor size and significantly increased the survival rate (Figure 1). Specifically, PLAG effectively reduced the A2BR level and the activity of the cRaf/Erk receptor-mediated signaling pathway, which is involved in tumor progression (Figure 1F–H and Figure 2A). PLAG has a unique mechanism of action through which receptor-mediated signaling is terminated by rapid receptor degradation after initial activation, unlike conventional antagonists that inhibit receptor activity (Figure 2C–G and Supplementary Figure 3). Increased A2BR/cRaf/Erk axis activation results in tumor overgrowth. However, PLAG reduced cRaf/Erk phosphorylation via A2BR degradation. As a result, tumor overgrowth was suppressed, and proliferation-related signaling pathways were terminated (Figure 4 and Supplementary Figure 1). As shown in Figures 2B, D, and 4E, A2BR ubiquitination and lysosomal activity were only increased in PLAG-treated cells.

Initially, PLAG induced the expression of TXNIP, a type of α-arrestin (Figure 3). The increased TXNIP protein level in PLAG-treated cells caused recruitment and co-binding of the NEDD4 and β-arrestin-1 complex to A2BRs (Figure 2C). According to recent reports, receptor binding to β-arrestin-1, but not β-arrestin-2, is induced by the NEDD4 complex [39–42]. Therefore, receptor binding of the α-arrestin family, which contains PpXy motifs, causes lysosomal degradation [43,44]. A2BR degradation by PLAG-induced TXNIP was confirmed in experiments using TXNIP-silenced cells (Figure 5). Failure of PLAG to inhibit tumor growth was observed in mice implanted with TXNIP-silenced tumor cells (Figure 6 and Supplementary Figure 4).

Another result of the ability of PLAG to promote receptor complex formation via TXNIP expression is the degradation of GPCRs, including PAR2, and epidermal growth factor receptor (EGFR) [35,36]. Internalized receptor degradation may ultimately interfere with the intracellular signaling of ligand-binding receptors.

Endosome signaling during intracellular GPCR trafficking is considered an essential pathway in cancer development and is of significant interest for drug discovery [45–48]. To date, the abilities of many GPCR signal-
modulating drugs to competitively inhibit ligand–receptor binding have been studied [49, 50]. The use of receptor antagonist strategies can cause substantial side effects due to the inhibition of critical signaling pathways essential for host defense. However, PLAG can attenuate excessive and continuous signaling through the degradation of activated receptors rather than the inactivation of receptors. This unique mechanism of action involves receptor degradation via the induction of TXNIP expression. PLAG has potential as a highly effective anti-tumor agent that can sufficiently replace antagonists, which can cause side effects and excessive secondary pathway activity by blocking signaling pathways through simple inhibition.

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Author contribution

Guen Tae Kim: Writing – Original draft, Methodology, Validation, Investigation, Formal analysis, Data curation Eun Young Kim: Investigation, Formal analysis Su-Hyun Shin: Investigation Hyowon Lee: Investigation Se Hee Lee: Investigation Ki-Young Sohn: Funding acquisition Jae Wha Kim: Project administration, Supervision, Conceptualization, Writing – Review & editing.

Declaration of Competing Interest

The authors declare no potential conflicts of interest.

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Corresponding author J.W.K. declares that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest. The authors thank Dr. Larry Kwak and Dr. Jeffrey Crawford for guidance and insightful discussions.

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