Abstract

Background: Pediatric cancers, particularly central nervous system (CNS) tumors, represent a significant cause of mortality among children in developed nations. Within this context, pediatric high-grade gliomas (pHGG) pose a formidable challenge. Diffuse intrinsic pontine glioma (DIPG), an aggressive and rare form of pHGG, primarily affects children ages five to ten and manifests in the ventral pons region of the brain. Treatment options for DIPG remain limited, with radiation therapy serving as the primary therapeutic modality. However, the efficacy of radiation therapy is hampered by adverse effects and the emergence of treatment resistance. To address these concerns, radiosensitizers such as CDDO-2P-Im assume a pivotal role. Acting as an enhancer of radiation therapy, CDDO-2P-Im augments its effectiveness by permitting the utilization of reduced radiation doses, thereby minimizing the potential cognitive consequences observed in pediatric patients. Additionally, manipulating the signaling pathway of the chemokine CCL2/MCP-1 offers a promising avenue for impeding glioma progression and augmenting immunotherapeutic responses. Synthetic oleanane triterpenoids, including CDDO-2P-Im, are currently being explored as potential inhibitors of CCL2/MCP-1, with researchers aiming to optimize the therapeutic outcomes of radiation therapy in DIPG while concurrently mitigating treatment-related toxicities.

Goals: Through rigorous research, our lab sought to test the efficacy of CDDO-2P-Im as a radiosensitizer through the use of combination therapy with radiation in a colony-forming assay. The hope was that the data would demonstrate statistical significance through a decrease in cell count with higher doses, which would illustrate an enhancement of the treatment.

Materials and Methods: To pursue the effects of CDDO-2P-Im in combination with radiation, cell harvesting of DIPG cell lines took place involving trypsinization; cells were detached from the culture and resuspended in a trypsin-inhibiting medium. Accurate cell counting was crucial for precise plating efficiency and survival calculations. Exponentially growing cells were plated into two new 12-well plates, incubated for adherence, and checked through imaging. Subsequently, cells were subjected to varying combinations of CDDO-2P-Im and radiation treatments (50nM, 1G, 50nM + 1G, 2G, and 50nM + 2G) or no CDDO-2P-Im treatment as a control group. The plates, including controls with no drug, were placed in a controlled incubator environment of 37℃ and 5% CO_2 humidity. After colony formation, cells were rinsed with PBS, stained with a glutaraldehyde-crystal violet mixture, and air-dried at room temperature. Following staining, cells could then be counted and statistically analyzed using imaging software.

Results: The results of the colony-forming assay showed a statistically significant decrease in both cell count and colony number in the radiation doses for which CDDO-2P-Im had been administered, indicating enhanced radiosensitivity of DIPG cells when subjected to the combination of CDDO-2P-Im and radiation therapy. This suggests that CDDO-2P-Im acted as a

radiosensitizer, augmenting the effects of radiation treatment and leading to a significant reduction in cell viability and colony formation.

Conclusions: Future objectives include conducting in vivo studies using animal models to determine optimal dosages and safety profiles, paving the way for potential clinical trials. Also, through exploring relevant biomarkers, research can lead to personalized treatment strategies, improving outcomes for pediatric DIPG patients and potentially revolutionizing pediatric cancer care and offering new avenues for combating this aggressive disease.

Background

In more developed countries, cancer is the most prominent cause of death among children. Specifically within the realm of pediatric cancers, central nervous system (CNS) tumors are the second-most prevalent, comprising approximately 40% of cancer-related fatalities. Unlike other pediatric cancers, which have experienced more advancements in prognosis, CNS tumors still remain difficult to treat, primarily due to pediatric high-grade gliomas (pHGG), which are aggressive tumors originating from glial progenitor cells within the CNS. A specific form of pHGG commonly affecting children between the ages of 5 and 10 is diffuse intrinsic pontine glioma (DIPG), a rare and aggressive form of pediatric cancer located in the ventral pons region of the brain that is more prevalent in males. The exact cause of DIPG is not completely understood; however, evidence suggests a potential association between its etiology and development of certain cells during cerebral tissue formation. One prominent molecular feature observed in approximately 80% of DIPG cases is the H3K27M mutation. This mutation affects the histone H3 gene and results in a reduction in H3K27 methylation, in addition to changes in other histone modifications, including increased H3K27 acetylation and H3K36 methylation. These alterations lead to tumorigenesis in DIPG and provide targets for therapeutic treatment.

Histologically, most DIPGs are classified as grade III or IV astrocytomas, with few resembling grade II tumors. The clinical course of DIPG is rapid, and patients often present with a variety of symptoms related to dysfunction in the brainstem. These symptoms can include diplopia, facial weakness or paralysis, long tract signs, ataxia, dysmetria, and dysarthria. DIPG is typically diagnosed through magnetic resonance imaging (MRI) of the brain, which illustrates characteristic features such as an increased signal on T2 FLAIR MRI sequences in the ventral

pons region. Contrast enhancement is usually absent, even though areas of necrosis may occasionally show enhancement. Diffusion-weighted MRI images can provide additional information about tumor characteristics and prognosis.A biopsy is a rarely-used alternative to the MRI, should the MRI not work.

Treatment options for DIPG are limited, and the prognosis remains poor. Surgical resection is generally not feasible due to the location and infiltrative nature of the tumor, so the primary treatment is radiation therapy. Steroids are also used to manage symptoms and stabilize neurological function. Obtaining a tissue diagnosis through stereotactic biopsy is crucial for classification, treatment planning, and research purposes. Clinical trials have explored various systemic therapies, but none have significantly improved survival rates. Hypofractionated radiotherapy has shown promise as an alternative to conventional fractionated radiotherapy. However, any form of radiation therapy in children carries a risk of neurocognitive effects, neuroendocrine dysfunction, hearing loss, vascular anomalies and events, and psychosocial dysfunction. Some tumors may also display characteristics of resistance to the radiation, which can be attributed to diverse biological modifications within the tumor and its surrounding microenvironment. These alterations encompass changes in cell cycle regulation, repopulation facilitated by cancer stem cells, hypoxia induction, modified oxidative stress management, evasion of apoptosis, altered DNA damage response and augmented DNA repair mechanisms, inflammation, and perturbed mitochondrial function and cellular energetics. These features allow the tumor to maintain mechanisms that can restimulate growth and enable it to survive through treatment. Due to these resistive tumor features, the use of radiosensitizers becomes pivotal in the treatment of pediatric high grade glioma (pHGG), including DIPG. By combining specific

radiosensitizer drugs treatments and radiation itself, tumor treatment has been improved both in vitro and in vivo. The proposed drug CDDO-2P-Im functions as a radiosensitizer to DIPG. The drug increases the efficacy of radiation therapy by allowing for lower doses of radiation and thereby helping maintain cognitive functions in pediatric patients.

Radiation therapy has been found to increase the production of tumor-associated macrophages (TAMs) and activated microglia, which leads to tumor recurrence. Playing a major role in this process and working to inhibit the radiation therapy's signaling of the chemokine CCL2/MCP-1 has shown promise in slowing glioma progression and enhancing the response to immunotherapy. When the chemokine interacts with its ligands, the chemokine receptors undergo structural modifications, prompting the binding of G proteins to specific regions within the receptors. This activation triggers a cascade of internal signals that lead to cellular movement and ultimately contribute to tumor expansion. Synthetic oleanane triterpenoids (SOTs), including CDDO-2P-Im, are currently being subjected to rigorous evaluation as potential inhibitors of CCL2/MCP-1 production and signaling to bolster support for radiation therapy for DIPG while also mitigating treatment-associated toxicities.

Hypothesis

Through the utilization of combination therapy on DIPG cells in cell culture through doses of radiation and CDDO-2P-Im, a decrease in cell count will be observed because 2P-Im will act as a radiosensitizer to enhance the effects of the treatment.

Methods

The process of harvesting cells from a donor culture involves trypsinization, which includes removing the medium covering the cells, washing them with PBS, and replacing the PBS with a trypsin-containing solution. The subsequent steps include removing the medium above the cells, washing the cells with PBS, and trypsinizing them to achieve a single-cell suspension. When the cells start to round up, indicating detachment, they are resuspended in a medium that inhibits trypsinization. Adding an appropriate volume of medium, supplemented with serum, neutralizes the trypsin solution. The cells are detached by gentle pipetting in the presence of the medium.

Cell counting is necessary to obtain precise data for plating efficiency in unirradiated controls or after specific treatments for survival calculations. Diluting the cell suspension to the desired seeding concentration and seeding the cells into flasks or plates is another crucial step that requires accurate dilution to ensure the correct number of cells are seeded.

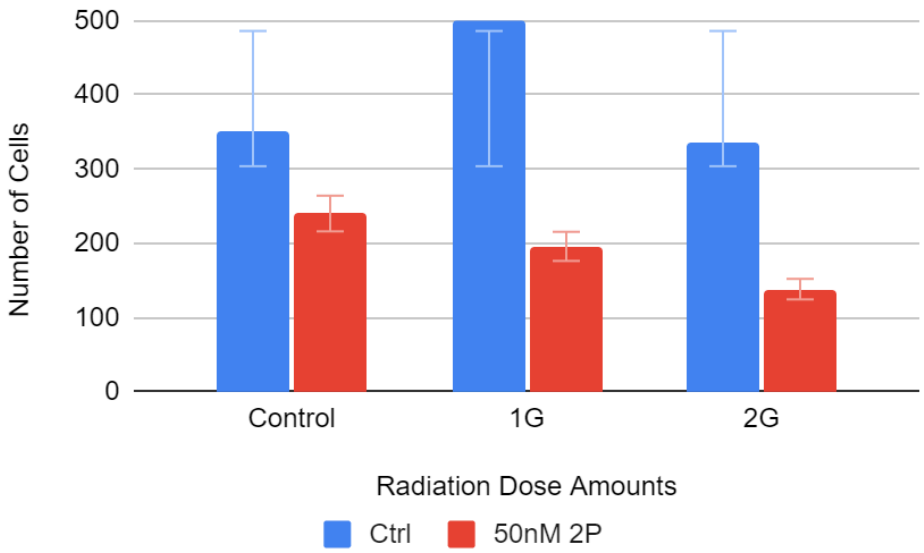
To begin the cell culture process, exponentially growing cells are harvested and replated in dishes or wells, with the number of cells depending on the desired treatment severity. In cases where the appropriate effect is unknown, different dilutions of varying cell numbers can be used. After replating, the cells are given time to attach to the plastic surface, which typically takes a few hours at 37°C. The attachment of cells is confirmed using a microscope. The cells in the dishes or wells are treated according to the requirements of the experiment. The treated dishes, in at least duplicate, are then placed in an incubator. The atmosphere within the incubator is adjusted to suit the growth medium's needs, such as maintaining excess CO_2 content. It is

important not to overlook humidity, so a tray with clean water is placed at the bottom of the incubator to prevent the culture medium in the dishes from drying up during incubation. The dishes remain in the incubator until the control dishes have formed sufficiently large clones. Clones are considered viable cells if they consist of more than 50 cells, which serves as the minimum count for viability.

After removing the growth medium above the cells, they are carefully rinsed with PBS. Then remove the PBS and add a mixture of 6.0% glutaraldehyde and 0.5% crystal violet, allowing it to sit for at least 30 minutes. Carefully remove the glutaraldehyde-crystal violet mixture and rinse the dishes or plates with tap water. It is important to avoid placing the dishes or plates directly under the running tap; instead, fill the sink with water and immerse them carefully. Leave the dishes or plates with colonies to air-dry at room temperature.

Results

A.



B.

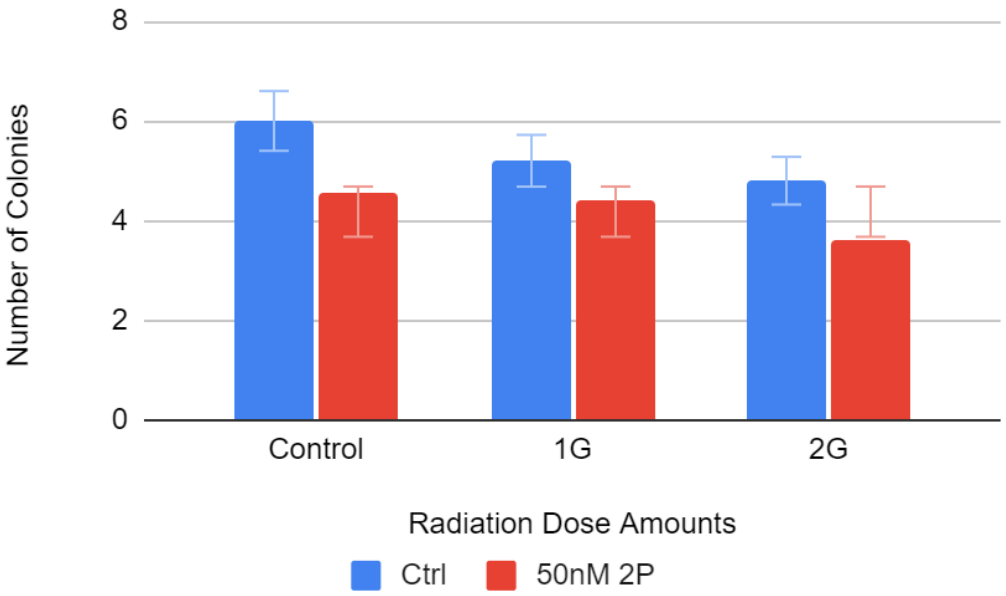


Figure 1. Statistical analysis depicting average values in graphical form of the contrast between varying doses of radiation with CDDO-2P-Im and without in regards to colony size and colony number.

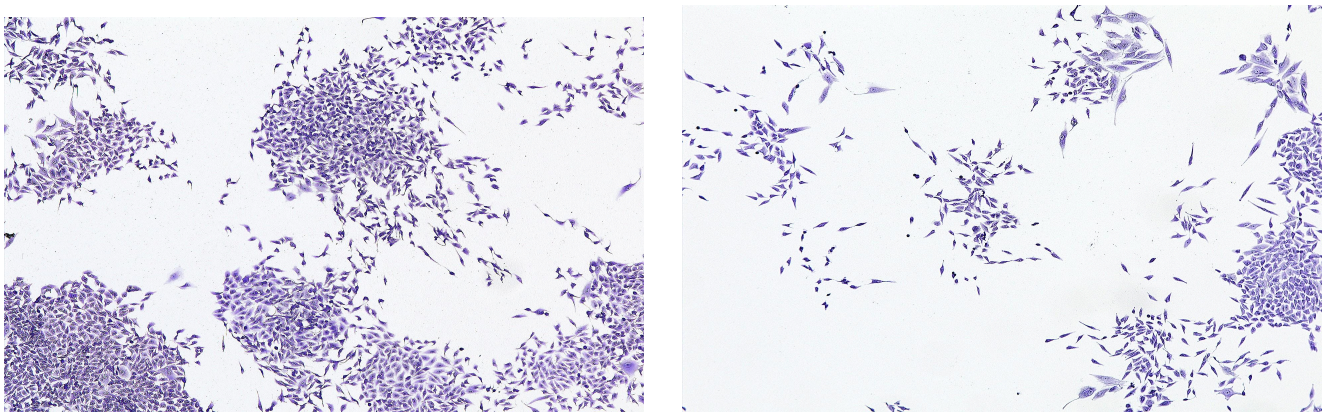


Figure 2. Microscopic depiction of the DIPG cells with the leftmost image depicting dosage of 1 Gy of radiation and the rightmost image depicting the combination of 1 Gy radiation and 50nM of CDDO-2P-Im

The colony-forming assay results unveiled a compelling and statistically significant decrease in cell count at higher doses of CDDO-2P-Im. This outcome signifies a pronounced enhancement in the radiosensitivity of DIPG cells when subjected to the combination of CDDO-2P-Im and radiation therapy. The observed reduction in cell count highlights the potential of CDDO-2P-Im as an effective radiosensitizer that is capable of augmenting the therapeutic response to radiation treatment in DIPG.

The successful findings from the colony-forming assay align harmoniously with the earlier sections of the paper, which established the challenges posed by DIPG as a rare and aggressive pediatric high-grade glioma. The limited treatment options and poor prognosis associated with this malignancy underscore the urgent need for innovative therapeutic strategies.

In the discussion of the molecular features of DIPG, the H3K27M mutation was highlighted as a prominent molecular alteration, driving tumorigenesis and offering potential therapeutic targets. The efficacy of CDDO-2P-Im as a radiosensitizer aligns with the endeavor to overcome treatment resistance observed in DIPG. By targeting tumor-associated macrophages (TAMs) and activated microglia, CDDO-2P-Im presents a promising approach to inhibit radiation-induced mechanisms that lead to tumor recurrence.

The combined treatment approach using CDDO-2P-Im and radiation therapy holds particular significance in the context of DIPG, where conventional therapies have shown limited effectiveness. By offering a means to improve radiosensitivity, CDDO-2P-Im addresses the challenges associated with tumor resistance and may reduce the reliance on high radiation doses,

potentially mitigating treatment-associated toxicities and preserving cognitive functions in affected children.

Conclusions. The investigation into the potential efficacy of CDDO-2P-Im as a radiosensitizer in conjunction with radiation therapy for the treatment of DIPG has yielded notable success. The outcomes of the colony-forming assay demonstrate a statistically significant decrease in cell count at higher doses of CDDO-2P-Im, indicating a substantial enhancement in the effectiveness of radiation therapy. These encouraging results highlight the promising role of CDDO-2P-Im in overcoming treatment resistance and minimizing the cognitive sequelae often observed in pediatric patients afflicted with DIPG. The successful findings from this experimental approach hold considerable clinical significance, offering a potential avenue to improve therapeutic outcomes for this rare and aggressive pediatric high-grade glioma. The ability to achieve enhanced radiosensitivity through the utilization of CDDO-2P-Im in combination with radiation has implications in optimizing treatment efficacy while reducing potential adverse effects commonly associated with conventional therapies.

Insights and advancements achieved through further research are imperative to translate these preclinical findings into clinical applications. In vivo studies employing appropriate animal models, such as murine models, will be essential in further elucidating the optimal dosage and safety profiles of CDDO-2P-Im in a more complex physiological context. These studies will allow researchers to discern any potential off-target effects and interactions, and to determine the most efficacious dosing regimen, which will ultimately inform future clinical trial design.

Beyond this, continued exploration of the molecular mechanisms underlying the radiosensitizing effects of CDDO-2P-Im can elucidate its interactions with cellular signaling pathways, further enhancing our understanding of its therapeutic potential. Additionally, investigating the combination of CDDO-2P-Im and other targeted therapies and immunotherapies may present an opportunity for synergistic treatment modalities, potentially circumventing resistance mechanisms and bolstering antitumor immune responses.

Efforts should also be directed toward identifying relevant biomarkers associated with treatment response, which could facilitate patient stratification and personalize therapeutic approaches in DIPG management. Such biomarkers may serve as predictive indicators of treatment efficacy, enabling clinicians to tailor treatments to individual patients, which will lead to improved outcomes and reduced treatment-related toxicities. The successful demonstration of CDDO-2P-Im as a potent radiosensitizer in this experimental setting signifies a pivotal advancement in the ongoing quest to address the challenges posed by DIPG treatment. By elucidating the potential of CDDO-2P-Im to augment radiation therapy, this research offers a promising avenue to improve patient prognosis and enhance the overall quality of life for children affected by this devastating disease. It is evident that continued research efforts, in collaboration with clinical trials, will be indispensable in harnessing the full therapeutic potential of CDDO-2P-Im and, ultimately, in transforming the landscape of DIPG treatment.

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