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**Design, Synthesis, and Evaluation of Novel Chlorambucil-Based Hybrids as Dual DNA/HDAC Inhibitors for Enhanced Antitumor Activity"**

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**ABSTRACT:**

Chlorambucil, a well-established alkylating agent, has long been used in the treatment of chronic lymphocytic leukemia (CLL); however, its clinical efficacy is often limited by drug resistance mechanisms, including DNA repair and glutathione-mediated detoxification. To overcome these limitations and enhance therapeutic potential, we report the rational design, synthesis, and biological evaluation of a novel series of chlorambucil-based hybrid molecules capable of dual inhibition of DNA and histone deacetylases (HDACs). These bifunctional compounds are engineered to retain the DNA-alkylating ability of chlorambucil while incorporating HDAC inhibitory moieties to induce epigenetic modulation and sensitize tumor cells to DNA damage.The hybrids were synthesized via a modular approach and structurally characterized by NMR and mass spectrometry. Their cytotoxicity was evaluated in vitro using leukemia cell lines and primary CLL cells. HDAC inhibition was confirmed by increased acetylation of histone H3 and H4, while DNA alkylation and cross-linking were quantified using comet assays and γ-H2AX staining. Furthermore, glutathione (GSH) levels and glutathione S-transferase (GST) activity were measured to assess cellular detoxification responses. Notably, the novel hybrids induced robust DNA damage and apoptosis even in samples with elevated GSH and GST, indicating partial circumvention of resistance pathways.These findings support the therapeutic promise of dual-targeting chlorambucil derivatives as more effective anticancer agents, especially in hematologic malignancies with established resistance to monofunctional drugs. Further in vivo studies are warranted to evaluate pharmacokinetics and therapeutic efficacy.

**Keywords**: chlorambucil hybrid, DNA alkylation, HDAC inhibition, chronic lymphocytic leukemia, glutathione, GST, drug resistance

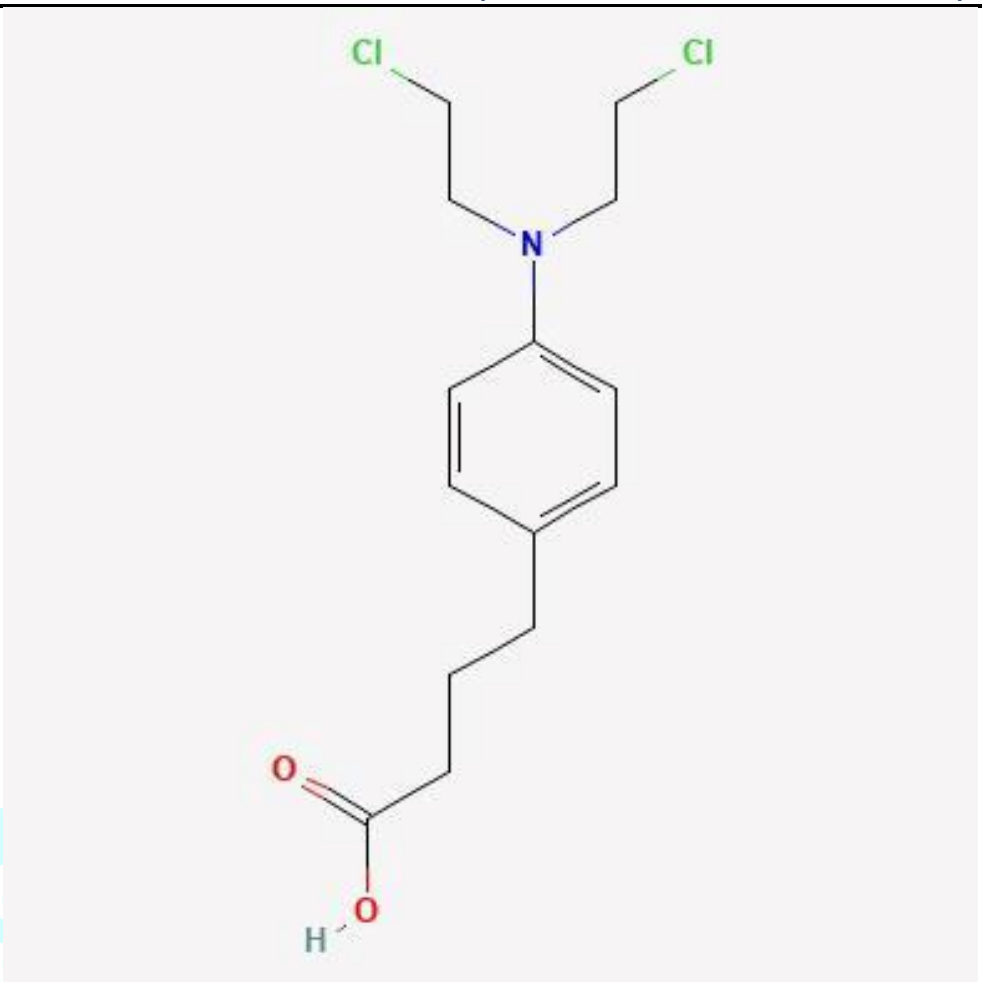



Fig.1.1 Structure of Chlorambucil molecule.

**DISCOVERY:**

In an attempt to produce less hazardous mustard gas derivatives for cancer treatment, the synthetic nitroge n mustard alkylating agent chlorambucil was created in the early 1950s.It was first used in clinical settings in 1953, mainly to treat chronic lymphocytic leukemia.Later, it was alsoused to treat other hematologic m alignancies, such as nonHodgkin's lymphomas and Hodgkin's diseaseThe phenylacetic acid component of chlorambucil, which is derived from mechlorethamine, increases its oral bioavailability and lessens advers e effects.Being one of the first oral medications toenable longterm cancer treatment with fewer side effects than its predecessors, its development represented a major advancement in chemotherapy. Chlorambucil was approved formedical use in the United States in 1957. It is on the World Health Organization's List of Essential Medicines. It was originally made from nitrogen mustard.

**FURTHER HISTORY:**

Nitrogen mustards arose from the derivatization of sulphur mustard gas after military personnel exposed to it during World War I were observed to have decreased white blood cell counts. Since the sulphur mustard gas was too toxic to be used in humans, Gilman hypothesized that by reducing the electrophilicity of the agent, which made it highly chemically reactive towards electron-rich groups, then fewer toxic drugs could be obtained. To this end, he made analogues that were less electrophilic by exchanging the sulphur with a nitrogen, leading to the nitrogen mustards. With an acceptable therapeutic index in humans, nitrogen mustards were first introduced in the clinic in 1946. Aliphatic mustards were developed first, such as mechlorethamine hydrochloride (mustine hydrochloride), which is still used in the clinic today. In the 1950s, aromatic mustards like chlorambucil were introduced as less toxic alkylating agents than the aliphatic nitrogen mustards, proving to be less electrophilic.

**PHYSIOCHEMICAL PROPERTIES:**

| PROPERTY NAME | PROPERTY VALUE | REFERENCES |
|---|---|---|
| Molecular Weight | 304.2 g/mol | Computed by PubChem 2.1 (PubChem release 2021.05.07) |
| XLogP3 | 1.7 | Computed by XLogP3 3.0 (PubChem release 2021.05.07) |
| Hydrogen Bond Donor Count | 1 | Hydrogen Bond Donor Count 1 Computed by Cactvs 3.4.8.18 (PubChem release 2021.05.07) |
| Hydrogen Bond Acceptor Count | 3 | Computed by Cactvs 3.4.8.18 (PubChem release 2021.05.07) |
| Rotatable Bond Count | 9 | Computed by Cactvs 3.4.8.18 (PubChem release 2021.05.07) |
| Exact Mass | 303.0792842 g/mol | Computed by PubChem 2.1 (PubChem release 2021.05.07) |
| Monoisotopic Mass | 303.0792842 g/mol | Computed by PubChem 2.1 (PubChem release 2021.05.07) |

. (Table:1.1)

**PHARMACOKINETICS:**

Absorption and Bioavailability

Oral administration of chlorambucil resulted in rapid and consistent absorption, with the parent compound detectable in plasma within 30 minutes. Preliminary data comparing oral and intravenous administration demonstrated bioavailability exceeding 70%, suggesting efficient systemic uptake.In contrast, administration of prednimustine, a prednisolone ester of chlorambucil, failed to yield detectable levels of chlorambucil or its key cytotoxic metabolite, phenylacetic acid mustard, in plasma. This indicates poor oral bioavailability of prednimustine, likely due to metabolic degradation or poor intestinal absorption.

Distribution and Metabolic Fate

Intravenous pharmacokinetic profiles of chlorambucil followed a two-compartment open model with first-order kinetics. Detectable and significant plasma concentrations of phenylacetic acid mustard confirmed active biotransformation and distribution of the alkylating metabolite, known for its cytotoxic properties.

Implications for Drug Design

The poor bioavailability of prednimustine underscores the limitation of esterified derivatives in achieving therapeutic systemic levels. Furthermore, the generation of phenylacetic acid mustard, despite its cytotoxic effect, raises concerns due to its lower therapeutic index. From a drug design perspective, these findings justify the development of novel chlorambucil-based hybrids with improved pharmacokinetic profiles. Dual-function molecules targeting both DNA and histone deacetylases (HDACs) should aim for enhanced tumor selectivity, improved systemic stability, and restricted metabolic activation to reduce off-target toxicity. These modifications should retain or enhance the DNA-alkylating potential while minimizing the formation of less selective or toxic metabolites.

## MECHANISM OF ACTION:

Mechanism of Action of Novel Chlorambucil-Based Hybrids as Dual DNA/HDAC Inhibitors for Enhanced Antitumor Activity. The novel chlorambucil-based hybrids are rationally designed to simultaneously target DNA and histone deacetylases (HDACs), combining the cytotoxic potential of DNA alkylation with the epigenetic modulation provided by HDAC inhibition. This dual-targeting approach is aimed at enhancing antitumor efficacy while potentially overcoming resistance mechanisms associated with monotherapy.

1.DNA Alkylation : Chlorambucil, a bifunctional alkylating agent, acts primarily through covalent binding to DNA. The nitrogen mustard moiety in chlorambucil forms highly reactive aziridinium intermediates, which alkylate the N7 position of guanine bases. This alkylation induces interstrand and intrastrand DNA cross-links, leading to DNA strand breaks, disruption of replication and transcription, and eventually cell cycle arrest and apoptosis. The DNA damage response pathway is activated, typically resulting in the engagement of p53-mediated apoptosis in sensitive tumor cells.

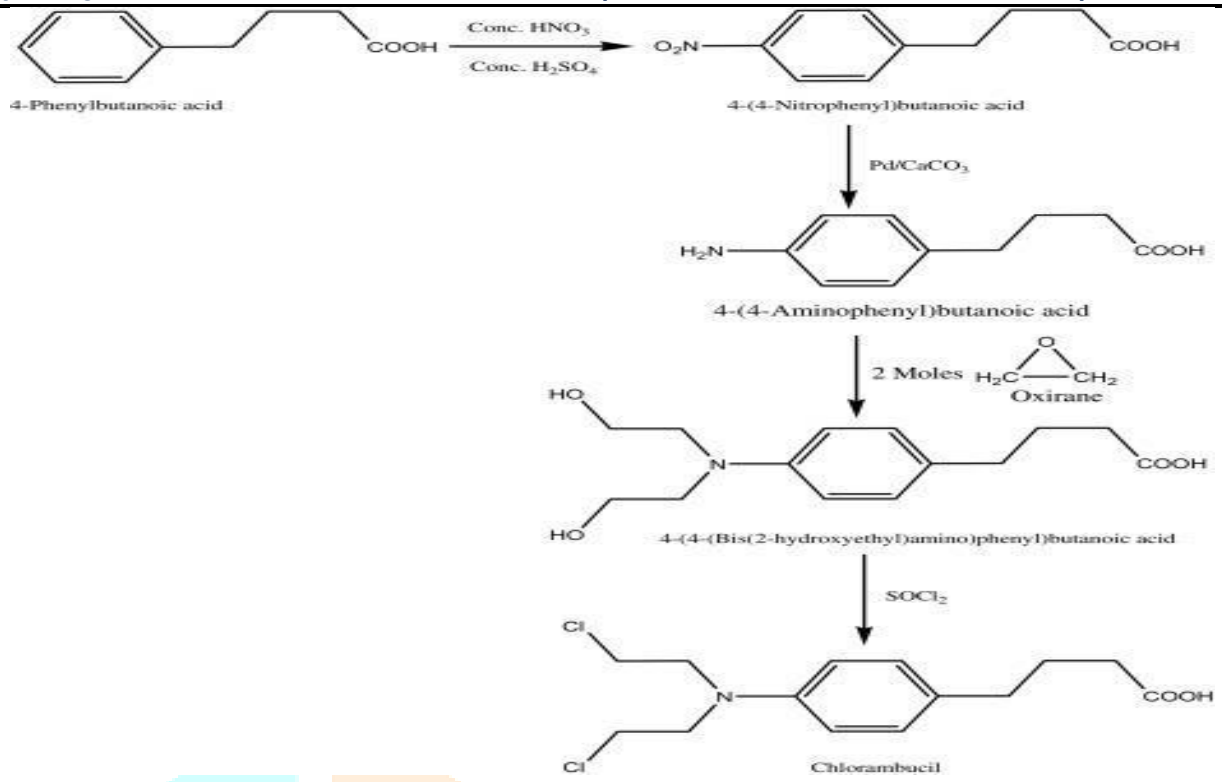
2.HDAC Inhibition : HDACs are a class of enzymes involved in the removal of acetyl groups from histone and non-histone proteins, leading to chromatin condensation and transcriptional repression. Overexpression or dysregulation of HDACs has been associated with oncogenic transformation, tumor progression, and resistance to apoptosis. HDAC inhibitors (HDACis) induce histone hyperacetylation, resulting in a relaxed chromatin structure that promotes the re-expression of tumor suppressor genes, cell differentiation, and apoptotic cell death.In chlorambucil-based hybrid compounds, the HDAC inhibitory moiety is covalently linked to the alkylating pharmacophore. This allows for co-localized activity within the nucleus: while the alkylating unit induces direct DNA damage, the HDAC inhibitory component modulates chromatin accessibility and enhances transcriptional responses to DNA damage.

3.Synergistic Antitumor Mechanism :The dual action of DNA alkylation and HDAC inhibition is hypothesized to exert synergistic cytotoxicity. HDAC inhibition enhances chromatin accessibility, facilitating DNA alkylation and crosslinking. Moreover, HDACis can impair DNA repair mechanisms, further sensitizing tumor cells to alkylator-induced damage. The re-expression of pro-apoptotic genes and cell cycle regulators through epigenetic modulation further supports a robust apoptotic response.

4.Structural and Pharmacological Considerations : Chlorambucil analogues have been chemically modified to optimize pharmacokinetics, improve cellular uptake, and minimize the formation of toxic metabolites such as phenylacetic acid mustard. Incorporation of stable linkers between the alkylating and HDAC inhibitory moieties ensures nuclear localization and preserves the functional integrity of both pharmacophores.These hybrids have shown improved antitumor activity in preclinical models compared to chlorambucil alone, with evidence of enhanced histone acetylation, DNA damage signaling, and apoptosis in cancer cell lines. The design further avoids the metabolic instability and low bioavailability

## METHOD OF SYNTHESIS:

The starting material for the synthesis of chlorambucil is 4-phenylbuanoic acid. First 4-phenylbuanoic acid undergoes nitration in the presence of Conc. HNO3 in acidic condition produces 4-(4-nitrophenyl)butanoic acid. Then 4-(4-nitrophenyl)butanoic acid undergoes reduction in the presence of Pd/CaCO3 to yield 4-(4-aminophenyl)butanoic acid which on reaction with 2 mol of ethylene oxide or oxirane to get 4-(4-(bis(2-hydroxyethyl)amino)phenyl)butanoic acid. Later the chlorination of compound in the presence of thionyl chloride yields the desired product of chlorambucil.




(Fig.1.2)

**MEDICAL USE:**

1. Division of cancer Chlorambucil is a chemotherapy drug that belongs to a class of medications called alkylating agents. It is primarily used in the treatment of certain types of cancer, particularly lymphomas and chronic lymphocytic leukaemia (CLL). Here are some details about the medical use of Chlorambucil:

2. Cancer Treatment: Chlorambucil is used in the treatment of various types of cancers, including Hodgkin's lymphoma, non-Hodgkin's lymphoma, CLL, and Waldenström macroglobulinemia. It is particularly effective in treating CLL, a type of cancer that affects the white blood cells.

3. Mechanism of Action: Chlorambucil works by interfering with the DNA replication process in cancer cells. It attaches to the DNA molecule and prevents it from unwinding and replicating, thereby inhibiting the growth cells.

4. Oral Administration: Chlorambucil is usually taken orally in the form of tablets. It is well-absorbed in the gastrointestinal tract, allowing for convenient administration.

5. Treatment Duration: The duration of chlorambucil treatment varies depending on the specific cancer being treated and the individual's response to the drug. It may be prescribed for short-term or long-term use, depending on the patient's condition.

6. Side Effects: Like other chemotherapy drugs, chlorambucil can cause side effects. Common side effects include nausea, vomiting, diarrhoea, loss of appetite, hair loss, bone marrow suppression (resulting in decreased blood cell counts), increased risk of infection, and an increased risk of developing other types of cancer in the long term.

7. Precautions: Due to its potential effects on the bone marrow, chlorambucil should be used with caution in patients with pre-existing bone marrow suppression or blood disorders. It is generally not recommended for use in pregnant women or individuals with severe liver or kidney disease.

8. Monitoring: Regular blood tests are often conducted during chlorambucil treatment to monitor blood cell counts and assess the drug's effectiveness. Dose adjustments may be made based on this result. It's important to note that the information provided here is a general overview, and specific details about the

use of chlorambucil should be discussed with a qualified healthcare professional. They can provide personalized advice based on an individual's medical condition and history.

**ADVERSE EFFECT:**

Chlorambucil, like other chemotherapy drugs, can cause several adverse effects. These side effects can vary in severity and may differ from person to person. Here are some common adverse effects associated with the use of Chlorambucil:

Nausea and Vomiting: These are common side effects of chemotherapy drugs, including Chlorambucil. Antiemetic medications may be prescribed to help manage these symptoms.

Diarrhoea: Chlorambucil can cause an increase in bowel movements and diarrhoea. It is important to stay hydrated and notify your healthcare provider if you experience severe or persistent diarrhoea.

Bone Marrow Suppression: Chlorambucil affects the bone marrow, leading to a decrease in blood cell production. This can result in low red blood cell counts (anaemia), low white blood cell counts (neutropenia), and low platelet counts (thrombocytopenia). These conditions can increase the risk of infection, fatigue, and bleeding. Regular blood tests are conducted to monitor blood cell counts during treatment.

Increased Risk of Infection: Due to the suppression of the immune system, individuals taking Chlorambucil may be more susceptible to infections. It is important to take precautions to avoid exposure to infectious agents and promptly report any signs of infection, such as fever, to your healthcare provider.

Hair Loss: Chlorambucil can cause hair loss, including scalp hair, body hair, and eyelashes. Hair usually grows back after the completion of treatment. Increased Risk of Secondary Cancer: Long-term use of Chlorambucil has been associated with an increased risk of developing other types of cancer, such as acute leukaemia.

Allergic Reactions: In rare cases, Chlorambucil can cause allergic reactions. Symptoms may include rash, itching, swelling, dizziness, or difficulty breathing. Seek immediate medical attention if you experience any signs of an allergic reaction.

TREATMENT OF OVERDOSE:

1. In the event of an overdose of the chemotherapy drug chlorambucil, immediate medical attention is crucial. Chlorambucil is a potent alkylating agent used primarily in the treatment of certain cancers, including chronic lymphocytic leukaemia and lymphomas. An overdose of this medication can lead to severe toxicity and life- threatening complications. The treatment of chlorambucil overdose primarily involves supportive care and measures to mitigate drug absorption and enhance drug elimination. Please note that the information provided here is for informational purposes only and should not replace professional medical advice. If you suspect an overdose or encounter any medical emergency, seek immediate medical assistance.

2. Emergency Medical Care: If you suspect a chlorambucil overdose or someone displays symptoms of an overdose, call emergency medical services immediately. Prompt action is essential to ensure the individual receives appropriate medical attention as soon as possible.

3. Symptomatic and Supportive Care: Once the patient is under medical care, supportive measures are initiated to maintain vital organ functions and manage symptoms. The following steps may be taken:Airway management and oxygen supplementation, if necessary, to ensure adequate oxygenation.Intravenous fluids may be administered to maintain proper hydration and blood pressure.Electrolyte imbalances, which can occur due to vomiting or diarrhea, may be corrected with

appropriate electrolyte replacement.Antiemetic medications can be given to control nausea and vomiting.

4. Gastrointestinal Decontamination: If the chlorambucil overdose occurred recently, gastrointestinal decontamination may be considered to reduce drug absorption. This can include: Inducing vomiting, preferably within the first hour after ingestion, if the patient is conscious and stable. However, induction of vomiting should not be performed if the patient has ingested caustic or petroleum-based substances, as it may lead to further damage.Administration of activated charcoal, which can bind to the drug in the gastrointestinal tract and limit its absorption.

5. Specific Antidote: As of my knowledge cutoff in September 2021, there is no specific antidote for chlorambucil overdose. Therefore, the focus remains on supportive care and general measures to enhance drug elimination.

6. Enhancing Drug Elimination Efforts to enhance chlorambucil elimination from the body may include: Hemodialysis: In severe cases of overdose or when signs of drug toxicity persist despite supportive care, hemodialysis can be considered. Hemodialysis can help remove the drug and its metabolites from the bloodstream.

7. Monitoring and Complication Management: Patients who have experienced a chlorambucil overdose require close monitoring of their vital signs and laboratory parameters. Chlorambucil overdose can lead to severe bone marrow suppression, resulting in anemia, thrombocytopenia, and leukopenia. Blood transfusions and growth factor support may be necessary to manage these complications and restore blood cell counts.

8. Psychosocial Support:Overdose situations can be emotionally distressing for the patient and their loved ones. Providing psychosocial support, including counseling and psychiatric evaluation, may be necessary to address any underlying issues that contributed to the overdose and prevent future incidents.

CONTRAINDICATIONS:

Chlorambucil should not be use in patients whose disease has demonstrated a prior resistance to the agent. Patients who have demonstrated hypersensitivity to chlorambucil should not be given the drug. There may be cross- hypersensitivity (skin rash) between chlorambucil and other alkylating agents.

INTERACTION:

1. Chlorambucil may interact with other medications, supplements, or substances, leading to changes in their effectiveness or an increased risk of side effects. Some potential drug interactions included.

2. Allopurinol: Concomitant use of Chlorambucil with allopurinol (used to treat gout) may increase the risk of bone marrow suppression.

3. Vaccines: Chlorambucil may reduce the effectiveness of live vaccines, and live vaccines should generally be avoided during treatment with Chlorambucil due to the risk of infection.

4. Immunosuppressive agents: The combination of Chlorambucil with other immunosuppressive agents may increase the risk of infections and immunosuppression.

5. Warfarin: Co-administration of Chlorambucil with warfarin (a blood thinner) may increase the risk of bleeding.

6. Phenytoin: Concurrent use of Chlorambucil with phenytoin (an anticonvulsant) may reduce the effectiveness of phenytoin.

7. Procarbazine: Combining Chlorambucil with procarbazine may increase the risk of pulmonary toxicity. Asparaginase: The concomitant use of Chlorambucil with asparaginase may lead to increased hepatotoxicity.

NOVEL MARKETD FORMULATION:

Novel strategies for cancer targeted delivery For effective cancer therapy, it is necessary to improve and develop novel strategies for effective delivery of chemotherapeutics to cancer cells. Conventional chemotherapeutic agents accumulate both in normal and tumour cells due to non-specificity. The goal of cancer therapy is to reduce the systemic toxicity and improve the quality of life. The landscape of cancer treatment has improved significantly over the past four decades. Direct drug administration may be associated with embolism, non-specificity and drug induced toxicity. Additionally, orally administered drug regimen is required to overcome biological barriers, protein binding and first pass metabolism to reach therapeutic concentrations in cancer cells. Direct drug administration into the tumour environment may be effective when the cancer is benign. However, the prognosis is completely different when tumours metastasize and invade surrounding normal tissues. Under such conditions (metastasis) tumour cells invade other organs by altering phenotype. Moreover, such cells over express efflux pumps (P-glycoprotein, MRP and BCRP) and metabolizing enzymes relative to normal cells. Such overexpression aids tumour cell survival and imparts resistance to xenobiotics (anticancer agents). For example, glioblastoma multiforme (GBM) is one of the metastatic diseases which is very challenging to treat till now. Delivery of anticancer agents to metastatic cancer cell at therapeutic levels is extremely challenging. Targeted drug delivery may counteract metastatic tumour and minimize off target toxicity. Targeted drug delivery may be achieved by exploiting overexpression of transporters and receptors on cancer cell plasma membrane. Also, ion channels such as potassium (K+), sodium (Na+), calcium (Ca+2), chloride (Cl-) and AQP4 channels may be targeted to regulate tumour metastases. Cancer cells, particularly GBM utilize these channels for migration.

CONCLUSION :

The development of novel chlorambucil-based hybrids as dual DNA/HDAC inhibitors represents a promising strategy for enhancing antitumor activity in cancers such as chronic lymphocytic leukemia and lymphomas. These hybrid compounds are designed to simultaneously target DNA replication and histone deacetylase pathways, aiming to overcome the limitations of conventional chemotherapy, particularly resistance mechanisms associated with monofunctional drugs like chlorambucil. Combining DNA-alkylating activity with HDAC inhibition may result in increased DNA damage, impaired repair processes, and reactivation of tumor suppressor genes through chromatin remodeling. Such dual-action compounds may also sensitize resistant cancer cells by modulating apoptotic pathways and altering the expression of genes involved in DNA repair, including those participating in homologous recombination and non-homologous end joining. Future studies should focus on optimizing the pharmacokinetic profiles of these hybrids, evaluating their specificity, and assessing their efficacy in both in vitro and in vivo cancer models. Furthermore, understanding the molecular basis of their antitumor mechanisms may guide the rational design of more potent and selective derivatives, potentially improving clinical outcomes in resistant cancers.

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