Biointerface Research in Applied Chemistry
Open-Access Journal (ISSN: 2069-5837)

Review
Volume 13, Issue 2, 2023, 161
https://doi.org/10.33263/BRIAC132.161

Nitrogen Mustard: a Promising Class of Anti-Cancer Chemotherapeutics – a Review

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Scopus Author ID 56754572800
Received: 25.01.2022; Accepted: 4.03.2022; Published: 29.03.2022

Abstract: The cancer tissue hypoxia regions are present with an acidic microenvironment that prevent the intake of various components like drugs, nutrients, and other metabolites, encouraging the resistance to various therapies. Chemotherapy, among all therapies, causes great off-target toxicity. Thus the focus of research is on the target drug delivery that ensures the efficacy, selectivity, and cytotoxicity within the tumor regions only. For this reason, many classes of chemotherapeutic drugs such as alkylating agents, antibiotics, antimetabolites, hormonal therapy, and other miscellaneous agents have been introduced. Although alkylating agents serve various sub-classes such as nitrosourea, nitrogen mustard, triazide, methanesulphonate, and ethylenimines, our review particularly focused on Nitrogen Mustard (NM). NM is one of the most widely used among all other classes since its high electrophilicity provides diversity in its reactivity and attachment to other synthetic and natural components.

Keywords: hypoxia; alkylating agents; nitrogen mustard; cyclophosphamides; DNA intercalating agents.

Abbreviations: NM- nitrogen mustard; ADEPT- Antibody directed enzyme prodrug therapy using nitrogen mustard; GDEPT- gene directed enzyme prodrug therapy using nitrogen mustard; VDEPT- virus directed enzyme prodrug therapy using nitrogen mustard; BDEPT- Bacteria directed enzyme prodrug therapy using nitrogen mustard; CNS penetrating drug- Central nervous system penetrating drug; ACTH- Adrenocorticotropic Hormone; ADH- Antidiuretic hormone; HIF- Hypoxia-induced factor; PET- positron emission topography; MISO- misonidazole; FMISO- Flourine-18 labelled misonidazole; EF5- 2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3, -penta-fluoropropyl)-acetamide; BOLD- Blood oxygenation level dependence; TOLD- Tissue oxygenation level dependence; MRI-Magnetic Resonance imaging; ^{18}F-FAZA- ^{18}F-flouroazomycin-arabinoside; HX4- ^{18}F-Flortanidazole; HAP- Hypoxia activated prodrugs; HPDCs-Hypoxia-activated prodrugs of diffusible-cytotoxins; HSCs- Hypoxia-selective cytotoxins; NADPH- nicotinamide adenine dinucleotide phosphate; DNA- Deoxyribonucleic acid; MGMT - O-methylguanine methyl-transferase; CBL- chlorambucil; FDA- Food and Drug Administration; DHEA- dehydroepiandrosterone; CP- Cyclophosphamide; CYP- Cytochrome P; 4-OHCP- 4-hydroxy-cyclophosphamide; ALDH- Aldehyde-Dehydrogenase; PAM- Phosphamide mustard moiety; IFO- Ifosfamide; DCE metabolite- De-chloro-ethyl; CAA- chloro-acetaldehyde; TRO- Trofosfamide; BRO- Bromofosfamide; IPM- Ifosfamide metabolite; SGLT- Sodium/glucose cotransporter; GLUT- Glucose transporter; IC- Intercalator; QM- Quinacrine mustard; Py3- Pyridine position-3; Pu5- Purine, position-5; APN- Aminopeptidase-N; TNF- tumor necrosis factor; CES2- carboxylesterase 2; SK/OV-3- Human ovarian cancer cell line; WiDr- Human colon carcinoma cell line; CFT073, HJ1020, and DH5α - Escherichia coli different cell lines; GST- Glutathione-S-transferase; MAP kinase- Mitogen activated protein kinase; TLK286- Telcyta; SM- Spiromustine.

1. Introduction

Irregular cell proliferation, invasiveness, and metastasis at different sites are the main indication of the tumor. Also, evidence has shown that tumor areas generally consist of an increased number of mitotic cells, tissue barriers break, the presence of incomplete or undifferentiated cells, and cells with large nuclei and prominent nucleoli. Other indications of tumor growth are tumor growth factor (autocrine stimulation or self-targeting), tumor-associated antigens, shedding of cell surface antigen, secretion of lytic enzymes like proteases, collagenases, lost or modified glycoproteins, and glycolipids, oncogene expression, altered surface charge density, increased amount of nucleic acid biosynthesis. In addition, neoplasm produces hormone-like substances, so-called paraneoplastic syndrome; this includes Hypercalcemia, Ectopic hormones like ACTH, calcitonin, ADH, and chorionic gonadotropin. Tumor cells contain genetic level changes or even alterations in the complete chromosome. The major hurdle in killing cancer cells is distinguishing them from normal cells [1].

Tumors are less dangerous when present only at the primary site of origin; once they break the barrier and invade the secondary site, the treatment rate becomes slow and difficult to deal with. It was believed that an adult malignant tumor generally takes 20 years to appear after the initiation by a disturbance in genes. The most common cancers in males are prostate, colon, and lung cancer, while in women include breast, colon, uterine.

1.1. Role of anticancer drugs.

Anticancer drugs are most effective in tumors such as the gastrointestinal tract, bone marrow cells, etc., with a high proliferation rate, but where the rate is slow (solid tumor), such as colon, breast, the lung is less susceptible to the treatment. Along with chemotherapy, radiotherapy and surgeries are also effective, but only in the case of the primary tumor; surgery causes maleficent intent to the secondary tumor by only festering the infection. In case of metastasis, chemotherapy becomes the major curative remedy, which could have its conditions work according to the type of neoplasm. Usually, the treatment methods are combined rather than alone for better effectiveness. When all three are used together, the method is called Adjuvant therapy.

1.2. Hypoxia: the challenge to all methods of therapy.

Hypoxia means "low oxygen level" in solid tumors surrounding the necrosis areas. In general, normoxic regions of cancerous cells and normal cells tend to resolve ischemia by increasing the blood flow to the affected area, increasing the oxygen supply. As the distance from the capillary increases, oxygen levels in tumor cells decrease, and resistance to chemo- and radiotherapeutics increases. But deep intercellular regions of tumor pack generally lack such regulation, leading to the formation of hypoxic regions. They settle the need for oxygen by forming hypoxia-inducible factors (HIFs), which causes a deleterious role by shifting cells to an anaerobic metabolic pathway followed by over-production of adenosine into the tumor matrix, and thus overwhelm T-cells activities [2]. The hypoxia region is depicted in Figure 1.

Hypoxia also causes upregulation in multiple gene expressions, resulting in angiogenesis, altered metabolic pathways, pH imbalance, and apoptosis as a response in tumor cells. Moreover, the essential role performed by these hypoxic cells is maintaining an adequate nutrient supply, causing recovery, and also vascular protection; thus, all the factors bolster

tumor growth and therefore resist the cells from any or all forms of therapies (chemotherapy, radiotherapy, surgery, photodynamic therapy, and sonodynamic therapy) [2,3].

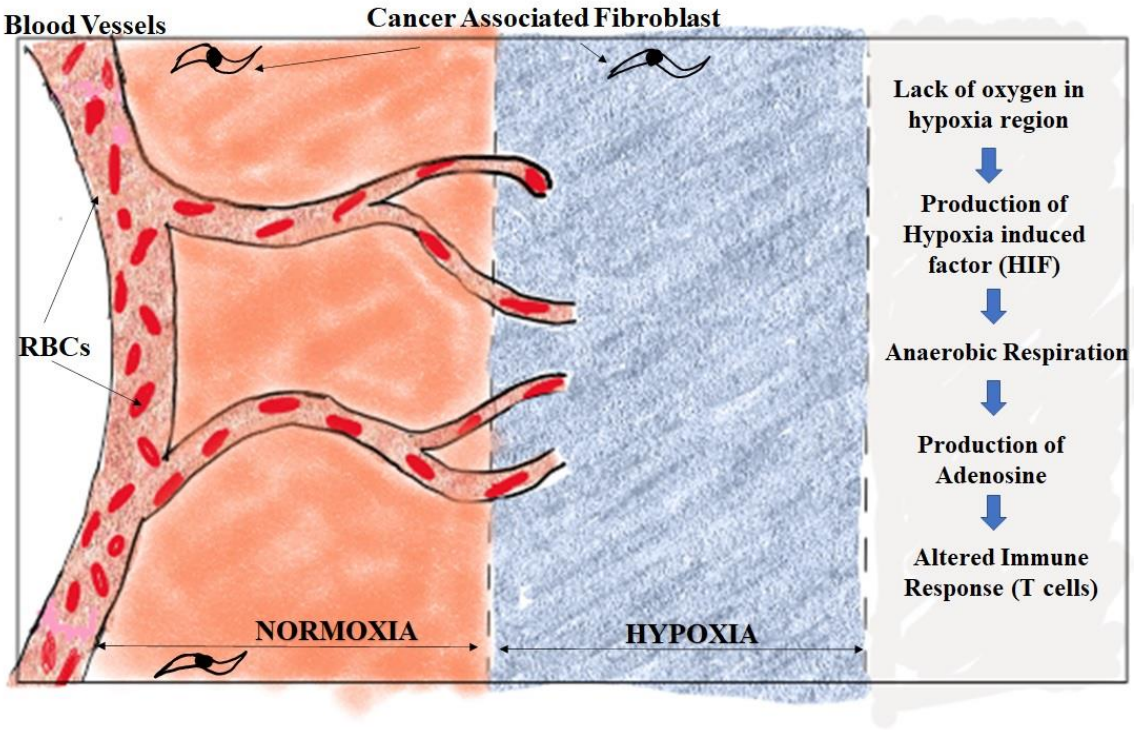

Figure 1. The figure depicted normoxia and hypoxia regions in tissue leading to the production of Hypoxia-Induced Factors (HIFs) and the formation of cancer-associated fibroblasts near the surface.

Hypoxia can be detected by both invasive as well as non-invasive methods. Polarographic electrodes, fiber-optic probes using Near-Infrared Spectroscopy, and immunohistochemical detection of exogenously administered drugs are some invasive methods. Positron emission tomography (PET) using hypoxia tracers (2-nitroimidazole), F labeled tracers (MISO, FMISO, EF5, C-acetate), MRI, blood oxygenation level dependence (BOLD), or tissue oxygenation level dependence (TOLD) MRI are non-invasive methods. Areas of hypoxia and necrosis melded together with a limit, or no tracer are unnoticed by low-resolution PET imaging. Thus novel tracers like FAZA, HX4, and F-pimonidazole have been used [4]. However, biomarkers are the approach used currently during hypoxia detection. BOLD and TOLD MRI are used as major biomarkers and are more robust than PET imagining as they do not require Radio tracers [2].

In 1970, an attempt was made to overcome the deteriorating effect of hypoxia by introducing a small molecule- Nitroimidazole- a molecule that can mimic the oxygen needed for the cells [5]. Trials for nitroimidazole derivatives were conducted along with radiotherapy, and the denouement shows minor but noteworthy improvement in the treatment at the local site. In the 1990s, technology achieved enough to capture oxygen measurements in a body, which certainly helped demonstrate the extent of hypoxia in tumor cells and the negative impact on radiotherapy, i.e., hypoxia limits the extent of radiotherapy efficiency. Currently, nimorazole (5-nitroimidazole) as a radio-sensitizer and radiation dose escalation is used in hypoxic tumors [4,6]. Hypoxia regions lack in p53 induced apoptosis [5], genes expression involved in drug resistance are also upregulated, thus favoring the growth of the tumor. Hypoxia activated prodrugs (HAPs) or bio-reductive alkylating agents were produced by using hypoxia as a principle for activating drugs. Nitroimidazole/nitroaromatics, quinone, aromatic

N-oxide, aliphatic N-oxide, and transition metal complex containing drugs are the major five classes of HAPs [3].

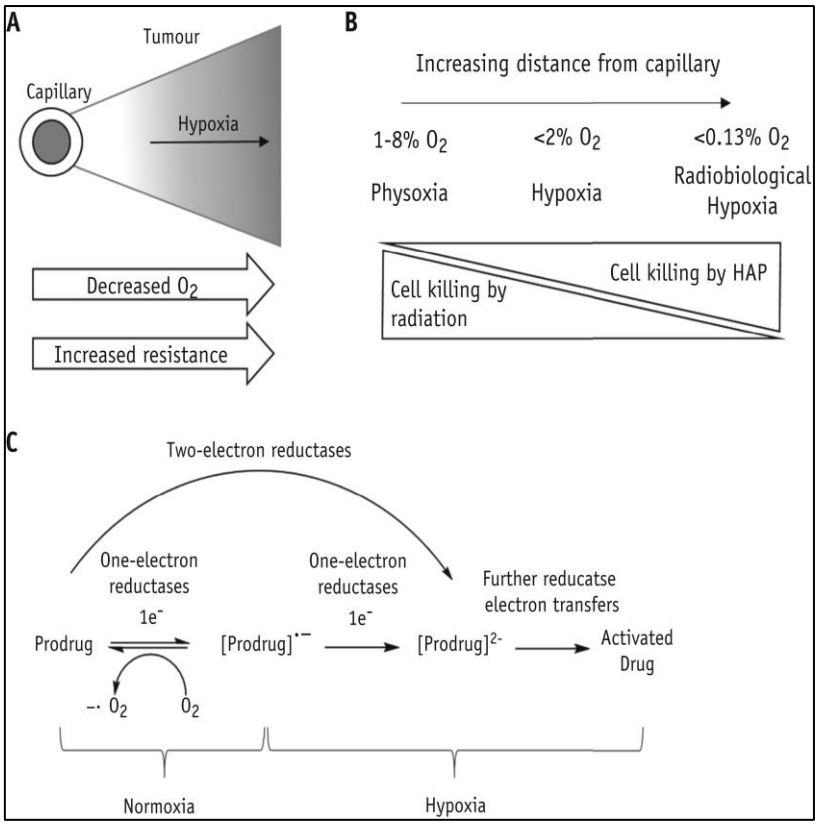

Figure 2. Hypoxia-activated prodrugs (HAPs) could target radiation-resistant hypoxic cells in tumors. (A) Illustration of tumor hypoxia. (B) Illustration of potential benefits of combining HAPs with irradiation. (C) The general mechanism of activation of hypoxia-activated prodrugs by 1 and 2 electrons (e^-) reductases. The figure used from International Journal of Radiation Oncology, Biology, Physics 2017, 981183-1196, http://doi:10.1016/j.ijrobp.2017.03.024 [9] under common creative license Creative Commons CC-BY-NC-ND.

The activation process (Figure 2) of Hypoxia-activated prodrugs of diffusible-cytotoxins (HPDCs) or Hypoxia-selective cytotoxins (HSCs) work under the principle of oxygen concentration in a cell. As we have studied so far that tumor cells contain regions of both normoxia and hypoxia, inactive prodrug undergoes 1-electron reduction (controlled by reduction potential of the compound) in both the regions by cellular enzymes, forming a radical prodrug anion. The normoxia region tends to suppress the further reduction by oxidizing the single-electron radical into the parent prodrug using dioxygen, but hypoxia regions are unable to perform such recovery, which causes the fragmentation of anion further through repeated reduction of radical to form 2-electron reduction active ion and then to its activated prodrug to exploit the hypoxia region. Therefore, it is essential that the activation occurs by single-electron route with oxygen sensitivity or follow oxygen-free double-electron reduction (reductase such as NADPH), otherwise affecting normoxia cells [7]. Also, the parent prodrug must show lower reactivity ab-initio, but the reduced ion should be highly reactive.

Moreover, many other approaches, such as hypoxia selective gene therapy, targeting the hypoxia-inducible factors (HIF1), etc., have been discovered [5,8]. Currently, the focus has turned to nanomaterials because these minuscule molecules tend to tune their size, shape, and surface area, thus reaching the hidden sites of the tumor cells; several nanocarriers have been developed; liposomes, drug-conjugate, nanogels, micelles [3]. Polymeric materials have also grabbed enough attention to perform these activities self-sufficiently.

2. Alkylating Agents

DNA consists of certain stable components such as phosphodiester linkages in the DNA skeleton with a half-life for spontaneous hydrolysis to be 3×10^7. However, the weak plugs are also present, which can easily undergo alterations. When left unrepaired, tend to react with other molecules such as aldehyde metabolites, reactive oxygen, and even water [10] which alter the DNA irreversibly.

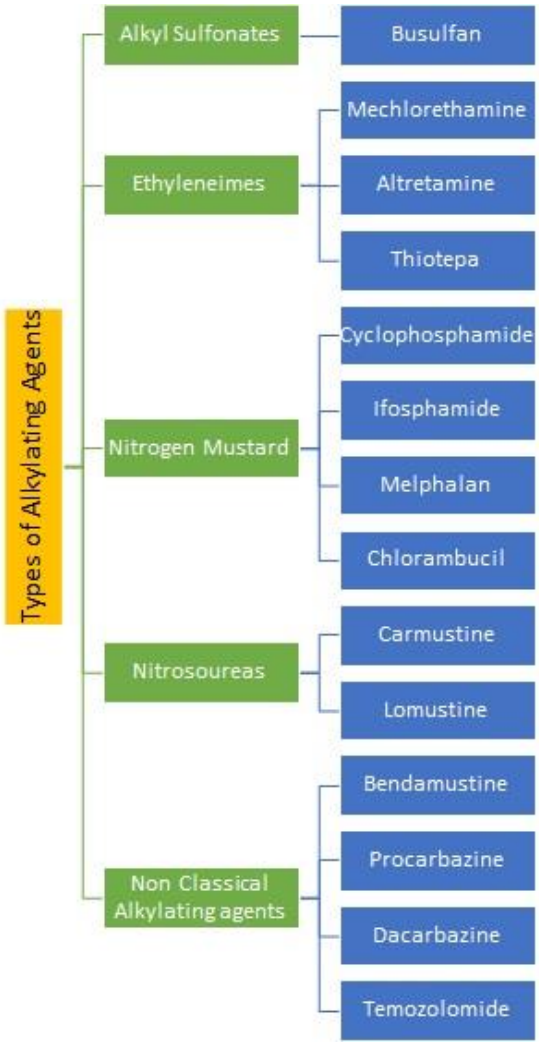

Figure 3. Classification of alkylating agents.

These mutations cause genomic instability leading to cancer and many more deleterious effects. Cancerous cells particularly become dangerous, and thus alkylating agents have been used to destroy these altered DNA-specific cells by unusual DNA crosslinking. These agents are electrophile that attacks the electron-rich DNA site within the cell; this results in the fragmentation of DNA, followed by the intra or inter crosslinking, mispairing disturbance, suspension of the DNA replication, and end by the cell apoptosis. These agents work effectively along with other agents, and their activity can be seen at every cell cycle stage [11]. Sometimes, cells show a reverse tendency toward anti-neoplastic drugs. For instance, O-methylguanine methyl-transferase (MGMT) could reverse DNA methylation by removing alkylating lesions, consequently resulting in the reoccurrence of the tumor O-benzyl guanine can inhibit MGMT by promoting the anti-neoplastic activity of the alkylating agents [12]. Moreover, modern inter-linking agents mainly alter the DNA healing pathways, such as BRCA1/2-deficient tumors [13], to cause permanent damage.

3. Nitrogen mustard as Alkylating Agent

Nitrogen mustard is an organic compound having bis(dichloro-ethyl) attached to nitrogen as the cardinal functional group. It was the first anticancer chemotherapy agent, found accidentally from the sulfur mustard after the first world war. It was 1946 when official trials for nitrogen mustard started for anticancer treatment. Since then, nitrogen mustard (NM) has been the host for modern alkylating anticancer curatives.

3.1. Mechanism of action of nitrogen mustard.

The lone pair of nitrogen in mustard causes the intramolecular cyclization (first-order, S_N1 cyclization) at neutral or alkaline pH resulting in the formation of a reactive intermediate ion called aziridium-cation (Figure 4).

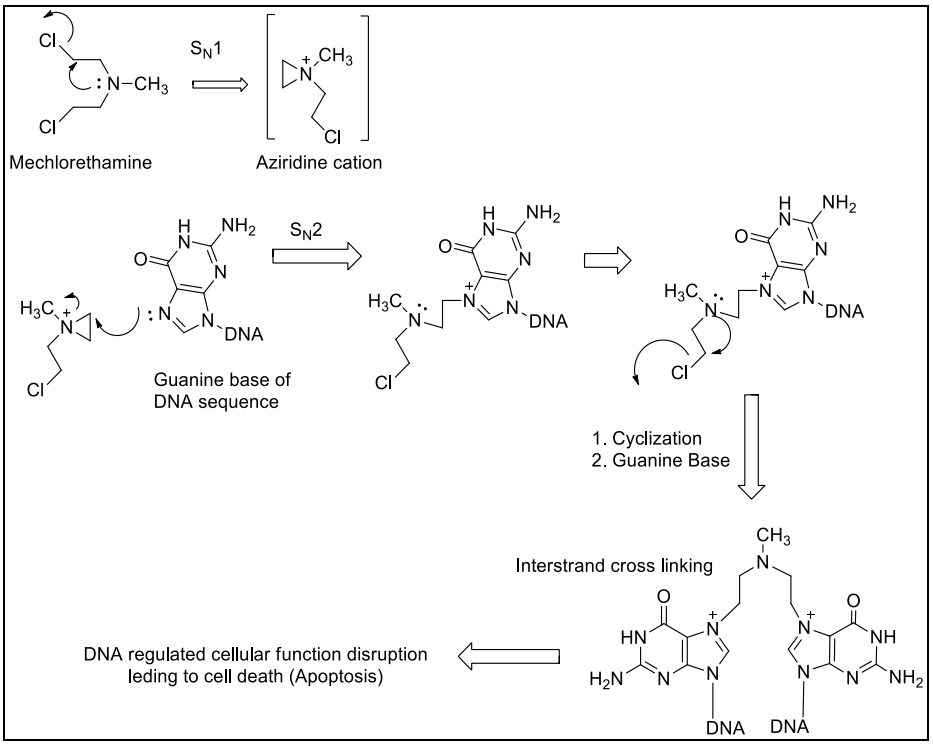

Figure 4. Mechanism of DNA intercalation between Guanine base pairs using mechlorethamine.

The ion is unstable and thus reactive enough to easily g*et al*kylated by the DNA molecule (nitrogenous base or phosphodiester linkage) to form a covalent bond, followed by the second displacement of chloride ion (S_N2), this causes the attachment of the second base of DNA (act as a nucleophile) to the crosslinking. It can occur repeatedly and causes the mispairing of nucleotides, leading to the intra or inter-cross linking in DNA strands, which is different from the original linking [14]. The cation tends to react with DNA at various sites such as N7-guanine, N3-adenine, N3-cytidine, and phosphodiester linkage or backbone of the DNA; nonetheless, guanine is the common site and also found to be the most toxic among all alkylation events [15]. Interstrand crosslinking has a low tendency to be formed (1-10% of total adduct) but is considered the critical lesion for the biomedical activity of nitrogen mustard [15]. After the first alkylation, the latter molecule is essential for determining the physical properties of the respective agent, such as reactivity, lipophilicity, transport, and distribution [11].

Moreover, the interaction of carrier and alkylating moiety affects the anti-neoplasm efficacy and reduces the side effects. The carrier constituent determines the pharmacokinetic

properties of the drug, such as absorption and its distribution *in vivo*. In addition, it also improves selectivity and activity and thus reduces the overall toxicity of the drug [16]. For instance, NM attachment to the benzene ring can reduce the electron density, particularly at the nitrogen atom, and consequently decrease toxicity.

3.2. Limitations and modifications.

During the very first trial for the drug on a real patient, it was observed that the NM is useful only in the first course of treatment; once the use is discontinued, the bone marrow recovers itself, and so does the tumor. On subsequent courses of treatment, the effectiveness further decreased to become nugatory. In addition, the major drawback was the lack of selectivity of the mustard molecule, i.e., normal cells or tissues also experienced great damage. The drugs caused blisters in some patients due to their cytotoxic reaction [11].

To overcome the counter effects, many analogs were formed in which the goal was to reduce the electrophilicity present in the mustard structure, which consequently reduces deleteriousness. Different eras of research on substituents linked to parent nitrogen mustard resulted in several classes, as depicted in Figure 3. Research over the last 70 years drags us to the new drug range for better target selectiveness called 'Prodrugs', which are produced to be activated inside neoplasm [14]. Along with prodrugs, targeting delivery of Nitrogen Mustard also contributed to an intensive extent for selectivity treatment [11].

Historic preview of NMs as alkylating agents with FDA approvals and use are tabulated in Table 1.

Table 1. Historic preview of alkylating agents (NMs) with FDA approvals and use [11].

S. no	Generation	Drug Name	Molecular formula	FDA approval	Use of the Drug	Ref.
1.	First	Mechlorethamine	C5H11Cl2N	1949	Mycosis Fungoid, Sezary syndrome, T-cell lymphoma,	[17,18]
2.	First	Chlorambucil	C14H19Cl2NO2	1957	Chronic lymphocytic Leukaemia, Hodgkin and Non-Hodgkin lymphomas, Breast and Ovarian Carcinomas, Mycosis Fungoid.	[17,19]
3.	Second	Uracil mustard	C8H11Cl2N3O2	1962	Breast cancer, Gastrointestinal, Leukaemia, colorectal cancer, pancreatic and adenocarcinoma.	[20]
4.	Second	Melphalan	C13H18Cl2N2O2	1964	Multiple Myeloma, Ovarian and Breast Cancer, Melanoma	[19]
5.	Second	Bendamustine	C13H21Cl2NO2	2008	B-cell lymphomas, lymphoplasmacytic lymphoma, Chronic lymphocytic Leukaemia, and recurring Hodgkin lymphomas	[21,22]
6.	Third	Melflufen	C24H30Cl2FN3O3	2020	Multiple Myeloma, myeloid leukaemia, Ovarian and Breast Cancer, Osteosarcoma, neuroblastoma.	[23,24]
7.	Steroidal	Estramustine	C23H32Cl2NO6P	1981	Prostate cancer, Palliative treatment of Carcinomas	[25]
8.	Steroidal	Prednimustine	C35H45Cl2NO6	NA	Ovarian, colorectal, Breast, Cervix, and Pancreatic Adenocarcinomas	[26,27]
9.	Phosphoramide	Cyclophosphamide	C7H15Cl2N2O2P	1959	Breast and Ovarian cancer, solid tumor in children,	[28]
10.	Phosphoramide	Ifosfamide	C7H15Cl2N2O2P	1987	Germ-cell testicular cancer, First-line treatment of sarcoma	[28]

3.3. Aliphatic mustard.

These groups are the first to be discovered to be linked with the nitrogen of the mustard drug and are thus commonly known as first-generation drugs. The most common type of group attached is the methyl group, and the drug was known as mechlorethamine nitrogen mustard.

3.3.1. Mechlorethamine.

The drug obtained its approval in 1949 by FDA [11]. It consists of a methyl group attached to the nitrogen of the mustard molecule [29]. It generates aziridinium ion (reactive species) as an intermediate that alkylates particularly N-7 of guanine molecule [14], resulting in mismatching pairing (Figure 4), DNA inter-strand crosslinking which ensue the suspension of repairing, growth, and synthesis of DNA, prevent cell cycle which denouement the cell apoptosis [30]. It has been used to treat Hodgkin's disease (stage III and IV), lymphoma, mycosis-fungoid, and lymphosarcoma [11,14]. Besides having side effects such as fatigue, nausea, vomiting, etc., the major downside was bone-marrow depression since the drug was not selective towards the tumor region [31]. It was suggested to avoid vaccination around the treatment with the drug. It cannot be taken orally as the reactivity of the drug leads to its reaction with water and results in its non-recovery inactiveness; thus, the only source is intravenously [11].

3.4. Aromatic nitrogen mustard.

With limitations to using aliphatic mustard, aromatic mustard was introduced with easy control by variations in the substituent on the ring [7]; for example, nitrobenzyl quaternary salts mechlorethamine, was considered an effective bio-reductive prodrug. The reduction potential of N-bis(2-chloroethyl)-N-(2-nitrobenzyl) ammonium chloride is enough (-358mV) [30] that it undergoes the activation process to form resultant products benzyl radical and mechlorethamine [7]. The advantage of using the electron-withdrawing aromatic ring is that it can deactivate the mustard molecule and enhance water solubility [7,30]. Mechlorethamine has an adequate half-life to back-diffuse to the surrounding neoplastic region at increased oxygen levels [11]. Several stable aromatic mustards (chlorambucil, melphalan, bendamustine) (using the same principle as above) were introduced. Their stability allows the drugs to be administered orally and thus classified as Second-order agents. Few important nitrogen-based alkylating agents are depicted in Figure 5.

3.4.1. Chlorambucil (CBL).

4-[bis(2-chlorethyl) amino] benzene butanoic acid (aniline derivative) or Chlorambucil drug was approved by FDA in 1957; it has been used as an effective anticancer drug since 1971 due to its binding ability to various forms of constituents such as antibody formed against neoplasm cells [32] or a carrier protein (for instance, transferrin) [33] present on the neoplastic cell surface, without altering the alkylating property of its chloroethyl constituent (nitrogen mustard) which is the basis for anticancer action [32]. The protein carrier approach was possible by introducing a maleimide group and its derivatives attached to the drug that has a tendency to be selectively attached to the sulfhydryl group of the carrier protein using the carbon double bond [33]. The biochemical approaches led to more effective drugs with enhanced selectivity towards tumor cells only with less toxicity due to the presence of a single carboxylic group in chlorambucil.

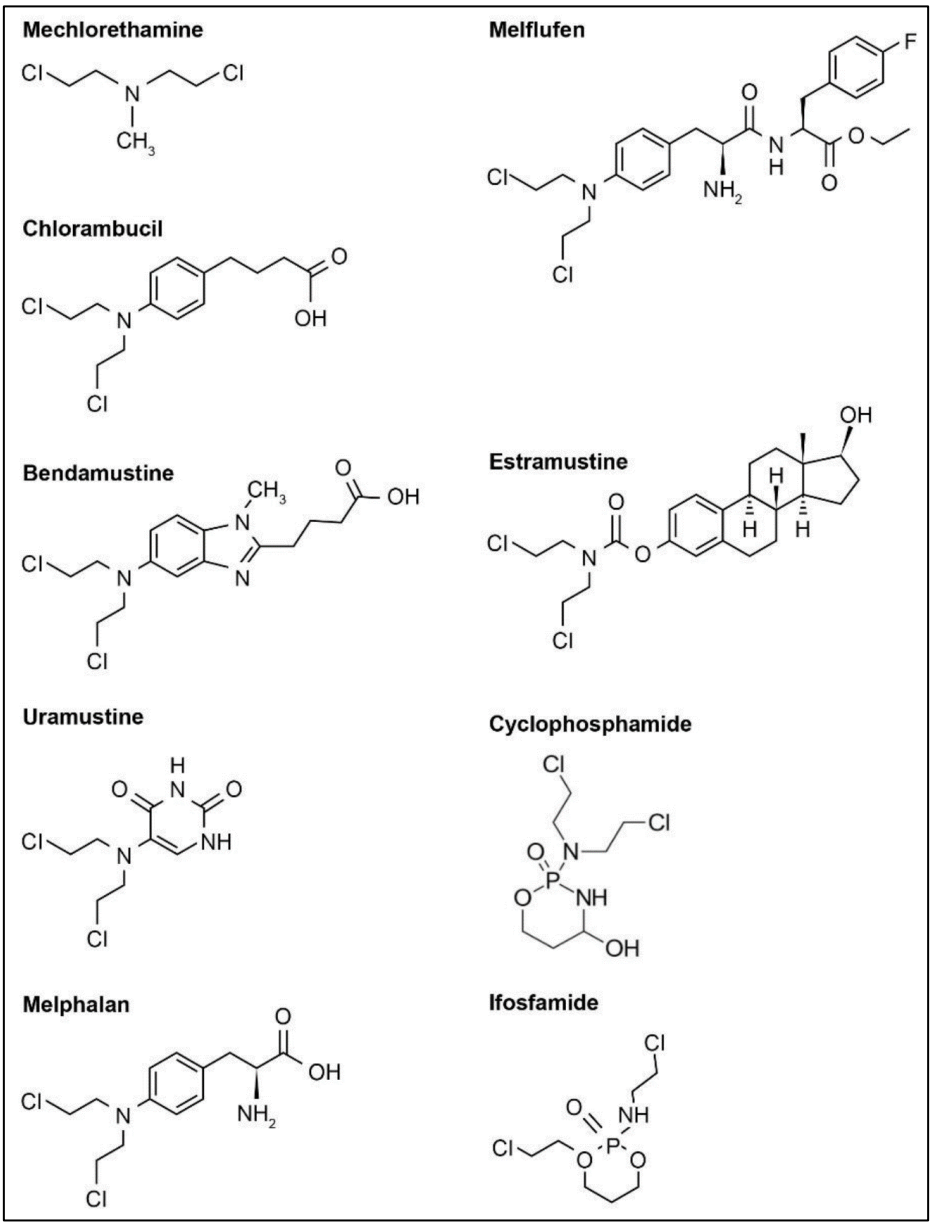

Figure 5. Evolution of Nitrogen-Based Alkylating Anticancer Agents. The figure used from Processes 2021, 9, 377. https://doi.org/10.3390/pr9020377 under common creative licence Attribution 4.0 International (CC BY 4.0) [11].

One such example is CBL ether lipid prodrug, where the liposomes are used as the drug carrier to the target tissue [34]. Secretory phospholipids are used as the moiety to attach the drug and lead to the formation of liposomes for transportation. These molecules are observed to be found in many types of tumor cells [34]. Previously, the oral administration led to the elimination of drug life within 1.5 h; these nanosized encapsulated conjugates of prodrug allow the resistance over metabolic action on the drug, also prevent drug leakage during circulation through blood, overall increasing the stability. Several polymer-based CBL prodrugs have also been introduced; the macromolecular side chain is attached to the drug-using imine, amide, or disulfide bond in order to prolong the drug loading [35]. Other advanced derivatives include dendrimers, inorganic nanoparticles, CBL-gemcitabine amphiphilic conjugate [36], the photo-controlled release of CBL with caged harmonic nanoparticles [37]. Chlorambucil has been used to treat lymphatic leukemia, ovarian and breast carcinoma, Hodgkin's disease, etc. The presence of an aromatic aryl ring ensures a slower interaction with cells compared to the first-generation drug [11].

3.4.2. Bendamustine.

N-methyl benzimidazole replaced the aromatic ring in chlorambucil to form bendamustine. The compound was approved by FDA in 2008 despite its discovery in 1960. The reason is its toxicity which disables its wide use in the beginning. Later, the duality of the drug, i.e., the alkylating and antimetabolite property, led to its use in chronic lymphocytic leukemia, non-Hodgkin lymphoma, and multiple myeloma. Although chlorambucil and bendamustine have similar mechanisms, solubility, and metabolism, benzimidazole makes the drug safer and more favorable. However, some of the repercussions experienced by patients include fatigue, headache, constipation, nausea, and adverse side effects, are lymphopenia, anemia, and thrombocytopenia [22]. Due to instability in water, nano-polymers based on highly efficient carriers for the drug have been used the meeting to the target.

3.4.3. Melphalan.

An aniline derivative, containing L-phenylalanine moiety attached to the mustard group, FDA approval in 1964 and since then used in palliative treatment for ovarian and breast cancer, and melanoma. The compound is administered intravenously and orally, depending on the dosing amount. Noticeably, it required a transport system that could be subsequently served by leucine, a naturally occurring amino acid that is overly expressed in tumor cells. Moreover, the active transport system buttressed the cellular uptake and enhanced its effect up to 3 cell lines [19]. Melphalan with quaternary ammonium moiety was also formed through an amide bond between them to enhance administration to the cartilaginous cancerous cells. Polyphosphoester link using polymeric conjugates of melphalan was also synthesized covalently, these polymers were made to be water-soluble, and they have shown negligible side effects.

3.4.4. Melflufen (peptide conjugate, Melphalan flufenamide).

It is third generation drug with FDA approval in 2020 consisting of ethyl-ester of the dipeptide of melphalan and para-fluoro-L-phenylalanine. Due to the high lipophilic property, the compound is readily taken up by uncommon cells (like myeloma cells) through the membrane barrier, causing the activation by the hydrolytic cleavage of the peptide bond using one of the several aminopeptidases, i.e., protein aminopeptidase-N (overexpressed in tumor cells and has proven role in neoplastic angiogenesis). The resultant alkylating moiety (melphalan) tends to enter the nucleus and lead to DNA disruption (Figure 6). The auspicious affinity of the drug towards solid tumors, multiple myeloma, and phase I/II trials led to its successful progress over time [30]. Also, it is believed that the drug is effective in third-line treatment on patients not exposed to any previous chemotherapeutic alkylating drugs and those who are melphalan exposed in previous treatment, also who are not suitable for transplantation [38].

In comparing melphalan and melflufen, the latter has several folds higher cytotoxic effects, increasing its efficacy with lower dosage intake and higher therapeutic activity, i.e., 30 min for the complete exposure inside the body. Also, the latter drug has enough efficacy to treat multi-myeloma cells where melphalan failed to participate actively. The higher intake of lipophilic-Melflufen due to gradient difference causes 50-folds greater melphalan exposure to DNA as compared to the use of melphalan directly [38]. Moreover, the modern melphalan-phenylalanine dipeptide derivative shows anti-angiogenic action and causes inhibition of cell

migration in multi-myeloma cells. Not only this, using Melflufen with various other drugs shows a great synergic activity toward tumor suppression [38].

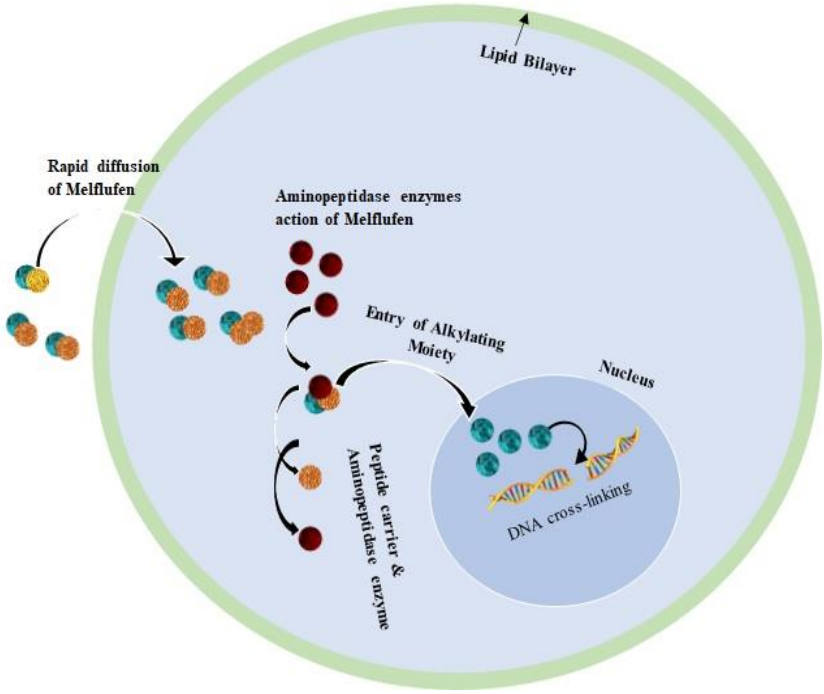

Figure 6. The mechanism of action of Melflufen, a peptide-drug conjugate, high lipophilicity of the compound ensures its rapid diffusion by easily crossing the neoplastic membrane. The peptidase enzyme present in the cell acts on the peptide-drug conjugate, which releases the alkylating moiety from the carrier. Nitrogen mustard moiety enters the nucleus, followed by DNA crosslinking.

3.4.5. Steroidal mustard.

The greatest challenge in chemotherapy is finding a selective drug approach that prevents toxicity and reduces the development of drug resistance. It is known that multidrug combinations can solve the latter deleterious effects. Nevertheless, show less effectiveness. Therefore, introducing an anti-neoplastic moiety attached to the steroid skeleton showed great progression due to their high selectivity and lipophilicity, which increased mustard moiety intake by crossing the lipid bilayer of the cells. Among several conjugates, steroid hormone receptor-mediated nitrogen mustard is successfully used to treat several hormone-dependent cancers: breast and ovarian cancer, prostate, the endometrium. For instance, dehydroepiandrosterone (DHEA)- nitrogen moiety alone shows negligible effect against neoplasm; however, forming steroid-DHEA drug shows a more effective response *in-vitro* and *in-vivo*. The reason behind this is the resemblance of functional groups in receptor (target protein) and the steroid drug moiety. The modification of steroid skeleton with amide or keto functions (lactam or 17B-acetamido group, or B-steroidal ring) [39] gives a great booster to the biological activity of the drug, as compared to the unmodified moieties of steroids. Some of the major drugs within the class are given below:

(i) Estramustine phosphate sodium: A modified-steroidal mustard where estradiol-carbamate-mechlorethamine combined. The compound is dephosphorylated on absorption and fragmented into estradiol, estrone, and estramustine as the major blood metabolites. The estradiol component increased the alkylating agent uptake in the target cells. It has been used in the palliative treatment and shows certain anti-androgenic activity. Certain adverse effects of the drug, such as hypertension, blood clots, and some allergic problems, affect calcium and

phosphate metabolism, along with certain risk factors; thrombosis, glucose intolerance, and vaccinations with live agents should be avoided. In addition, the drug causes anti-microtubule assembly as one of the major drawbacks since the fragments of the drug in the blood are attached to the microtubule protein and tubulin, which as a result, causes separation of the microtubules [40].

(ii) Prednimustine: The drug was prepared by the ester of prednisolone (corticosteroid) and chlorambucil. It shows both alkylating as well as corticosteroid properties. Acute leukemia, mammary carcinoma, lymphoid leukemia are diseases where the drug is generally used [27].

(iii) Homo-azasteroid (lactam steroids): This type of drug is formed by the attachment of lactam moiety (cyclic amide group) to the esterified form of the steroid-alkylating drug. It is become efficacious in several neoplastic tumors and leukemia both *in vivo* and *in vitro*. For instance, Lactandrate and Lactestoxate showed great anti-cancerous results against colon carcinoma. However, the latter showed better results than Lactandrate [41]. In 2016, Trafalis *et al*. [42] reported positive results with four modified forms of lactam steroids.

3.4.6. Phosphamide nitrogen mustard.

Phosphoramide mustards (1,3,2-oxazaphosphinane) are formed by the conversion of the base nitrogen mustard into non-toxic prodrugs and then actively transported into cancer cells where they are enzymatically converted into their active, cytotoxic forms [11]. Phosphamide NM has various classes like cyclophosphamide, Ifosfamide, Mafosfamide, Trofosfamide, Bromofosfamide and Glufosfamide to name a few. The importance of each subclass and mode of action are discussed hereof.

(i) Cyclophosphamide (CP): The compound has been widely used as a therapeutic agent in various cancer treatments. Instead of phosphamidase enzyme action, the liver encourages the cytochrome family (CYP450; CYP2B6, CYP2C19, CYP2C9, CYP3A4/5) activation of the drug by hydroxylation of CP. The parent molecule undergoes hydroxylation to form two disparate molecules: 4-hydroxy-cyclophosphamide (4-OHCP) and tautomer aldo-phosphamide. The prior molecule forms an inactive carboxy-cyclophosphamide in the presence of bio-catalyst Aldehyde-Dehydrogenase (ALDH), whereas the latter follows a different path.

Most of the Aldo-phosphamide is converted into carboxy-cyclophosphamide in the presence of an elevated concentration of ALDH in certain accelerated proliferating cells such as bone marrow stem cells, liver, and intestinal epithelium and therefore protect these cells. However, a small quantity of this tautomer molecule undergoes β-elimination to form the desirable phosphamide (PAM)-mustard moiety and a by-product, acrolein. Acrolein could cause bladder inner surface damage and a few other severe effects. Figure 7 gives a detailed insight into the metabolic pathway of cyclophosphamide.

CP can be taken orally and intravenously as well; intravenous intake allows the complete exposure of metabolic molecules in the blood within 2-3 h followed by elimination half-life of 3-12 h; it has been observed that regardless of considerable bioavailability, 10-25% of the drug abandoned with urine without being altered. Moreover, there are several repercussions during the treatment with CP, such as vomiting, anorexia, abdominal discomfort or pain, alopecia, diarrhea, and sometimes jaundice. Other severe negative concomitants include leukopenia and anemia; however, these are reversible once the dosage is reduced. Germ cells production can also alter, resulting in sterility in sexes. Lastly, the long-term use of cyclophosphamide resulted in secondary cancer risk [43].

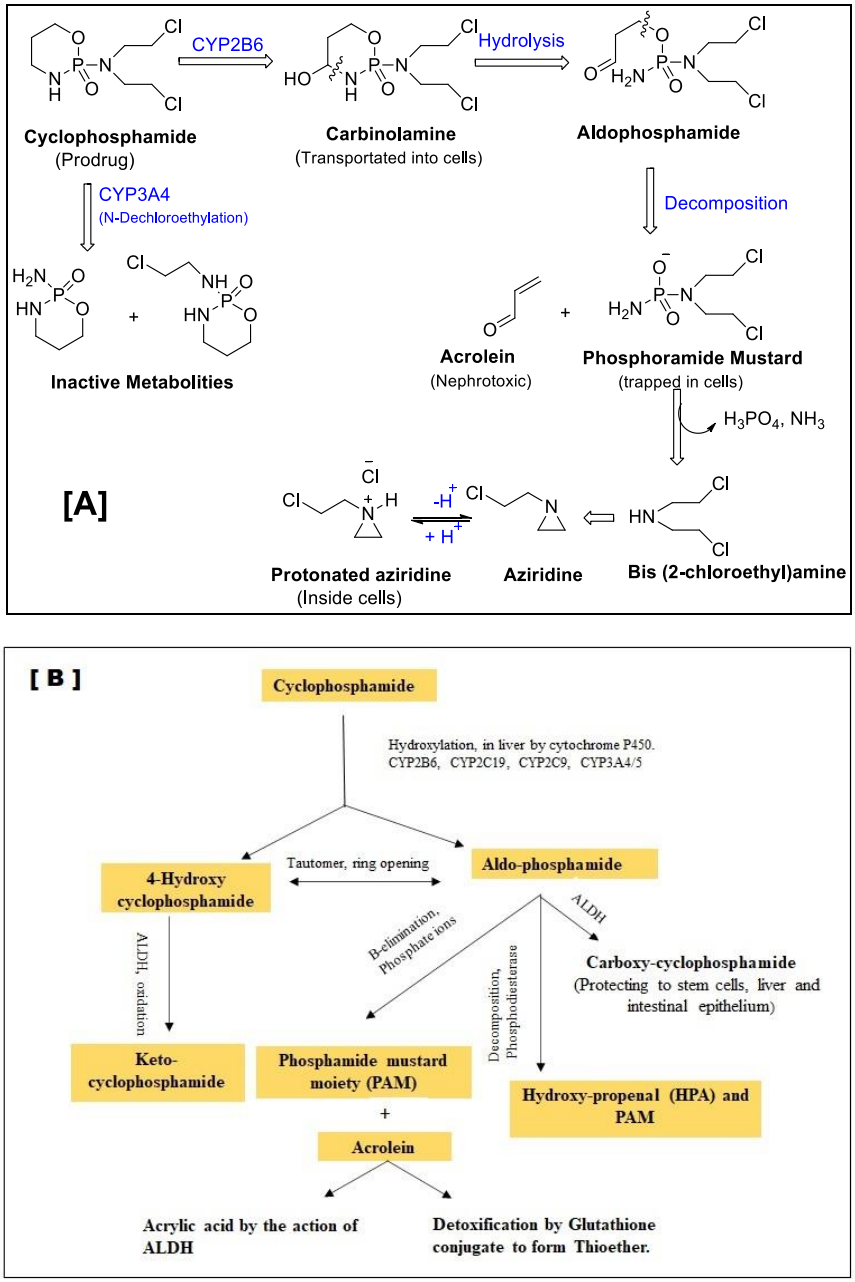

Figure 7. Metabolism of Cyclophosphamide [**A**] Structural representation; [**B**] Outline of the metabolic pathway. Initiated by the Cytochrome enzyme in the liver to form 4-hydroxycyclophosphamide and aldo-phosphamide as intermediates which further follow different pathways such as β-elimination to form a mustard molecule of phosphamide and acrolein. In the presence of Aldehyde Dehydrogenase (ALDH), 4-hydroxy cyclophosphamide, as well as carboxy-cyclophosphamide, converted into keto and carboxy-cyclophosphamide, respectively.

(ii) Ifosfamide (IFO): An isomeric cyclophosphamide (having chloroethyl group attached to the N-atom of the cyclic ring), used in several anti-neoplastic treatments such as testicular, cervical, lung, ovarian, and breast cancer, non-Hodgkin lymphoma, osteogenic and soft-tissue sarcoma. Ifosfamide produces 7-atom DNA cross-links (compared to 5-atom cross-links with cyclophosphamide) [40]. The functioning of the drug depends utterly on the metabolism. Cytochrome 450 monooxygenase is responsible for the hydrogenation of the compound and fragmentation of the parent drug to acrolein and the desired phospharamide-mustard molecule. Acrolein binds to some protein molecules and is thus responsible for the cytotoxic effect. The isophosphoramide (IPM) moiety shows the alkylating property by damaging and altering the usual DNA linking. Both cyclo-PAM and Ifosfamide are

hydrogenized by common CYP450-2B1 and 2C6/2C11. However, CYP450-3A activated the isomeric form only [40]. Additionally, the above usual metabolism competes with another pathway, i.e., deactivation (oxidation) of the chloro-ethyl group. Oxidation causes the removal or elimination of one of the chloro-ethyl groups or even both groups, leading to the synthesis of de-chloroethyl (DCE) metabolites. These metabolites include 2-DCE-Ifosfamide, 3-DCE-Ifosfamide, 2,3-Didecholoroethylifosfamide, and almost an equal amount of chloro-acetaldehyde (CAA) [44]. CAA affects the body by causing neurotoxicity, urotoxicity, and sometimes cardiotoxicity. It was observed that 50% of the curative medicine administered is consumed by this unwanted pathway [40]. In comparison to cyclo-PAM, Ifosfamide caused less leukopenia effect and showed lower cross-resistance. Encephalopathy is also a major consequence related to oral administered dosage only.

(iii) Mafosfamide: Mafosfamide is a stable synthetic 4-OH CP derivative containing thiol group. The presence of sulfur-hydroxyl substituent (mesna - Sodium 2-mercaptoethane-sulfonate) on the parent CP molecule protects against prop-2-enal (acrolein) induced toxicity without interrupting CP anti-neoplastic activity. N-methyl -Mafosfamide, a derivative, was known to perform chemically stable and biological oxidative prodrug [45]. The compound undergoes rapid demethylation in a highly pH-dependent (7.4 pH of plasma and 37°C temperature) medium to form imino-CP by the removal of mesna substituent; the reaction is followed by the addition of water molecule to form 4-OH CP, which is subsequently ended by the formation of PAM-mustard molecule and prop-2-enal. The entry of the drug itself and its metabolite 4-OHCP across the cell is through passive diffusion. It was believed that the mesna group is converted into the free thiol group in the kidney, which would trap acrolein molecules and decrease hemorrhagic cystitis and other urotoxicity. Although the drug is similar to CP, it doesn't need activation by the hepatic enzymatic system [45]. Generally, antimetabolites (such as gemcitabine, methotrexate, cytarabine) are used in intrathecal administration; mafosfamide is added as an effective agent in the list since its administration showed well tolerance and also no signs of neurotoxicity.

(iv) Trofosfamide (TRO): It has been approved to use as one of the anticancer drugs since 1973. Similar to its congeners, the metabolism of TRO occurs inside the liver by either of the two pathways, i.e., 4-hydroxylation and N-dechloroalkyalation; both are NADPH dependent [46]. IFO and CP are the two metabolites formed by side-chain oxidation of TRO upon the action of the liver biocatalyst: CYP450. Besides the several cDNA-expressed biocatalysts, CYP3A4 and CYP2B6 are the two main enzymes responsible for both pathways. It was investigated that the role of cytochrome biocatalyst-3A4 is dominant over biocatalyst-2B6, and thus tumors with significant CYP-3A4 release experienced a more effective TRO remedy. The compound has enough lipophilicity to cross blood-barrier at ease and is generally hydroxylated at a higher rate than IFO [46]. Moreover, the TRO has been used in the I and II-line treatment of soft tissue sarcomas and palliative treatment in non-Hodgkin lymphoma with a response rate of about 50-80% at oral daily doses of 150mg [40] with low deleterious effects.

(v) Bromofosfamide (BRO): An IFO analog, having bromine substituent. Its action involves crosslinking DNA strands or deoxyribose-protein crosslinks and alkali-labile sites in a concentration-dependent manner in HeLa cells [47]. The substituent allows the compound to be administered more towards the crosslinking with macro-molecules. Also, its potency is higher than IFO metabolite; isophosphoramide mustard (IPM). Moreover, IFO was more prone to cause urotoxicity than BRO. The oral intake caused a faster absorption.

(vi) Glufosfamide (β-D-glucoseisophosphoramide-mustard): It was believed that the glucose uptake in tumor cells is much greater than in normal cells, and thus on this principle, the glucose molecule is glycosidically linked to the Ifosfamide metabolite (IPM) to form a new drug- Glufosfamide with greater effectiveness than IFO [48]. The mechanism of entry of the drug into the abnormal cells is active transmembrane transportation using SGLT1-3 and GLUT1-5 transporter [47] (genes responsible for glucose transport in the cell); among these, the co-transporter SGLT3 (Na-dependable transporter) plays quite a major role. Phlorizin and Phloretin are the inhibitors of these transporters and resultantly decrease the drug intake and action. Moreover, liver cytochromes are not required for metabolism. IPM-glucose attachment increases the stability of the drug; the increased glycolysis and glucose intake are some cognitive signs of tumor cells only and subsequently increases the drug selectivity. Later, the rationale for the increased glucose uptake turned out wrong, and the drug was a failed attempt to improve oxazaphosphorine cytostatic [49].

3.5. Target delivery using mustard moiety.

Nitrogen mustard drugs have regioselectivity particularly dominant at N-7 of guanine in the major groove due to the lowest electrostatic potential at the site [50]. Moreover, the accumulation of Glutathione (a low-molecular thiol present in the cell) causes resistivity towards alkylators. Therefore, the DNA-directed intercalation encounters these drawbacks, effectively carrying the same mustard moiety. Due to higher selectivity towards specific sequence crosslinking in DNA, intercalators cause greater gene-toxic monoalkylation and consequently reduce the carcinogenic side effects of alkylators [51]. Major and minor groove occurs from the anti-parallel arrangement of the helix in the DNA. The difference arises due to the behavior of amino sites at different base pairs, such as in major-groove of B-DNA, C6 amino site of Adenine or C4 amino site of cytosine can act as H-donating whereas N7-Ad, O4-Thy, N7, and O6-Gu base pair can act as H-accepting group along with thymine-methyl as a hydrophobic site. The minor groove of B-DNA consists of N3-Ad, O2-Thy, N3-Gu, O2-Cyt base pairs as H-acceptors and the C2-amino group of Guanine as H-donor [30]. These groves are essential in determining the combing of binding proteins during the process of transcription as well as replication.

3.5.1. DNA intercalation.

Intercalation has become one of the prominent antitumor curative methods since alkylation of DNA occurs more rapidly with a change in the pattern of the targeting site. For instance, certain Intercalators (ICs) alkylate N3 and N1 of adenine at the minor groove and inter-groove, respectively. Small molecular ligands or intercalators were sandwiched between the two adjacent DNA base pairs to cause H-bonding disturbance (through hydrophobic interaction). This resulted in helical unwinding, which disturbed DNA and its dependent bioprocesses. The ICs binding reversibly but tightly to the chromophore component attack the action of certain enzymes and relieve the strain over the DNA during transcription and replication. These base displacement molecules are mostly planar, aromatic, and polycyclic [52]. One of the oldest classes of IC is Acridine, a planar molecule with a suitable potency to act vector for drug delivery at tumorous sites [53]. The mustard derivatives of acridine ensure attacking the DNA with both the alkylating agent and the acridine molecule itself and,

therefore, enhance the drug's activity with greater selectivity. Acridine and even anthraquinone work impressively in antileukemic treatment.

One such Acridine derivative is quinacrine mustard (QM), which behaves as a fluorescent probe in response to its interaction with biomolecules (proteins). The QM can cause intercalation before the covalent interaction with N-7 of Guanine. Among all the mustards, QM shows huge diversity in guanine base pairs. Moreover, the simultaneous activity of QM is only possible when the site of intercalation is 3' of the site of alkylation. For intensive reaction, the presence of Guanine or Thymine immediately 3' to the reacting G is preferred [54]. The binding freedom of chromophores could be limited by methoxy or choro groups on the chromophore moiety [55].

For 9-aminoacridine (9Aa) chromophore, the preference of attack is py3'-pu5'base steps, also the cytosine and 5'-GCpu possible [55]. During the attachment of the chromophore to the mustard molecule, the length of the alkyl chain is a determining factor since the short chains show certain rigidity, which constrains the aniline mustard molecule at a right angle to the 9-Aa and leads to the attack at N7 of guanine (5' end GT), in addition to this, the substituents $(CH_2)_2S$ and $(CH_2)_2O$ at major grooves participate in H-bonding which further enhances the reactivity at N-7 of guanine. The increase in length provides flexibility to shift the molecular attack towards 5' A-T at the N7 adenosine site regardless of the presence of a linking group [55].

Quinazoline (Qz) is quite a weak intercalator; It shows the same properties as 9-Aa in the case of guanine intercalation; however, different for adenine. With different substituent groups such as Qz-$(CH_2)_3$, it weakly gets connected and alkylates the N-adenine in the major groove side. However, In Qz-B$(CH_2)_3$, the chromophoric moiety spares down the alkylation due to steric hindrance.

The presence of magnesium ions could alter the selectivity in sequence by all three given drugs. Only some portions of the drugs are available for DNA intercalation since a major part undergoes hydrolyzation, which DNA itself could catalyze. Several different derivatives have been identified as potent DNA direct intercalator chromophores, such as 9-anilino-acridine, 9-anilino-quinoline, anthraquinone, and its derivative cyclo-pent-anthraquinone.

3.5.2. Minor grooves (MG).

The sequential selectivity of the intercalators (IC) is quite low, and thus minor groove agents have successfully canopied them. The aromatic annulate structure of the agent easily fits with the minor groove curvature. Also, the positive charges attract the negative tunnel of the groove. Lastly, their tendency to act as H-exchanger makes them a much better carrier approach, indeed stabilizing the overall DNA. These agents show minimal limitations by mustard moiety due to their high sequence and regioselectivity. The 4-Anilino-quinoline quaternary salt with aniline mustard family is A-T specific minor groove ligands that alkylate N3 site of both guanine and adenine at 3' ends of A-T rich sequences [50,56]. However, the weak aqueous solubility determines the activity and dose potency of the compound. Oligopyrrole derivative antibiotics, such as Netropsin and Distamycin A [50], are studied intensively since they are naturally occurring A-T specific ligands. The alteration in the aromatic ring of oligopyrrole results in the formation of a synthetic derivative, Lexitropsin. The compound has high specificity toward long DNA sequences. Similarly, several poly-pyrrole derivatives were introduced with attachment to different mustard moieties. The attachment of

the type of mustard and the number of pyrrole-amide units both affect the alkylation pattern in the DNA.

Poly-imidazole derivative of Lexitropsin is accepted as GC selective minor groove agent. The chlorambucil-containing MG analog showed high cytotoxicity with less dosage use than the unmodified chlorambucil. This also ensures the reduction in damage to the neighboring sites.

Poly-benzamide attached to mustard molecule reported to alkylate at N3-Ad site in Poly A sequence and at 5' T-A and 5'A-T sites [50]. Impressive to be noticed that the compound uses two distinct mono-alkylating mustard molecules, which are essential for the highest cytotoxicity.

9-anilinoacridine-4-carboxamide is a potent regioselective MG agent due to molecular asymmetry. The attachment of aniline mustard to the 9-anilino ring showed minor groove alkylation at N3-Ad up to 57% and N7-Gu adduct in a small portion. The chromophore behaves as a DNA-winding agent, presenting the aniline side chain to the minor groove and carboxamide to the major groove.

3.5.3. Antibody-directed enzyme prodrug therapy (ADEPT) using nitrogen mustard.

ADEPT is an appreciable approach to delivering a high concentration of drugs at the tumor site. It is cell-cycle independent, dose-reliance, and less resistance-causing technique. It is mainly an extracellular activation of the prodrug. The mechanism of interaction between the drug to the tumor site is multistep. Firstly, monoclonal antibodies associated with antigens present only on the tumor cell surface are used as a vector to carry the enzyme. An optimal time is provided for the unbound antibody-enzyme conjugate to be cleared from the blood by accumulated at the antigen of the tumor site. The second step is to administer an inactive drug activated by the enzyme and attacks the tumor site.

Fragment secondary antibodies, F(ab)2, is the choice of the antibody as a delivery vehicle since it has a great ability to localize quickly at the tumorous surface and the clearance from the blood is quite fast. The prodrug is an essential component, it must show linear dose-cell killing relation, and therefore alkylating agents are the most successful among all classes. Furthermore, the resistance that occurred against alkylating class can be easily overcome by increasing the dosage, and the enhancement doesn't cause toxicity with respect to the targeted therapy. There are certain ligands attached to the prodrug to increase the overall selectivity and therapeutic effect—for instance, galactose is attached to the Doxorubicin as an activation initiator [57]. After degradation of the ligand by β-*galactosidase* enzyme, it also released florescent signals for tracking its pathway inside the body. Other ligands such as biotin (present in Gemcitabine prodrug), folate (present in Paclitaxel and folate-tubulysin prodrug), hormone ligand is also available [58]. For tracking and imaging, sometimes light response labeled moieties are also used in prodrugs. Isothiocyanate is one example. In addition, polymers and nanoparticles are also tethered to the prodrugs to overcome traditional limitations by unmodified prodrugs. Albumin [57] and cathepsin B-degradable copolymer [59] are a few examples. One limitation to consider is the leakage of active drugs from the site to the blood, possibly corrected by selecting the active drug with a short half-life.

In general, the prodrug would be expressed anywhere near the desirable enzyme, and therefore, the choice of an enzyme became the crucial factor. Because enzyme substrate has more stable bonding and is less susceptible to hydrolysis, the overexpressed enzyme can break the linkers and activate the prodrug [57]. There are certain drawbacks when selecting an

enzyme, such as its efficiency to degrade the bonding and release active drug, heterogeneity in gene expression results in the subclass of tumors with consequently different enzyme expression, and some enzymes expressed in tumor and normal tissues. All these factors could only deteriorate the drug efficacy. Therefore, only a few enzymes are efficient for the target release.

One of the successful ADEPT systems consists of F(ab)2 vector carry carboxypeptidase G2 enzyme obtained from non-mammalian pseudomonas RS-16. It can effectively attack the carcinoembryonic antigen, followed by the administration of Di-iodophenol mustard glutamate prodrug (4- [N, N-bis(2-iodoethyl) amino] phenoxy-carbonyl L-glutamic acid, ZD2767P) [60]. The prodrug, in the presence of the enzyme, effectively changes to the di-iodophenol mustard active metabolite along with glutamate moiety [60]. It has proven to be worked successfully in both cytotoxicities and growth inhibition.

APN (aminopeptidase N) is a commonly found enzyme and a widely used biomarker, drugs related to APN like Ubenimex, Tosedostat, NGR-TNF, tTF-NGR, and J1 are under clinical trials [57]. β-galactosidase, β-glucuronidase, CES2 (carboxylesterases), caspases-3, Cathepsins, Dt-Diaphorase, Histone Deacetylases, Legumain (or asparaginyl endopeptidase), Glucarpidase (CPG2) are some enzymes that have a fully active role in various cancer. With many important factors, carboxypeptidase (COP) G2 was one of the best-suited enzymes. Researchers have recently formed a prodrug-carbon quantum dot with the enzyme-cleavable linker. The drug could perform highly efficient multiple roles.

3.5.4. Gene-directed enzyme Prodrug therapy (GDEPT) or suicide gene therapy using Nitrogen mustard.

The technique successfully overcomes conventional chemotherapy by selectively killing neoplastic tissue only, like ADEPT. However, the delivery components used in GDEPT are different from ADEPT since the site of action is different. Here, cancer cells are introduced by the specific gene vector or exogen encoded enzyme, followed by the prodrug administration. The inactive drug is activated by the enzyme intracellularly. Three factors to be considered for GDEPT; the great affinity of prodrug towards the specific enzyme and low for other enzymes, the gene encoded for the same specific enzyme, and lastly, the easy diffusion of the prodrug across the membrane of the target well as neighboring tumor cells. An enzyme non-occurring in mammalian class, absent or in low concentration in neoplastic tissues, i.e., viral (adenovirus and retrovirus are most common) or bacterial enzyme, is usually the selection choice since familiar enzymes can easily express at off-target sites. However, mammalian enzymes are also possibly used [61]. The usual approach used here is to enhance the enzyme behavior from the natural substrate towards the prodrug or change its characteristics, such as greater stability and low immunogenicity [62]. The ideology for prodrugs to be efficient is a sufficient half-life for the bystander effect (killing of neighboring cancerous cells by the indirect effect of prodrugs from transgenic cells) but not long enough to enter the blood circulation [62]. The bystander effect prevents cancer deterioration or ensures tumor regression.

The first gene-encoded carboxypeptidase G2 (CP-G2) GDEPT system was introduced by Marais *et al.* [63]. Its natural substrate is folic acid but has the ability to catalyze the hydrolysis of mustard prodrugs. The lipophilicity allows its transportation across the cell membrane. His observation demonstrated that only 12% of CP-G2 expressed cells were able to cause total kill in SK/OV-3 and WiDr cancer tissues [63]. The above system was later improved by Marais *et al.* [64]. They have proved the efficient role of using the same enzyme

as an exoenzyme (tethered on the tumor cell's surface) rather than direct administration intracellularly. This was demonstrated by the fact that prodrug activation using exoenzyme occurred interstitially, which ensures its uniform distribution among the local cells, consequently ensuring a stronger bystander effect. Also, the technique does not require cell-to-cell communication, such as gap junctions, which are usually absent in tumor cells [64].

However, the major drawback faced by the group is the strong immune response once the enzyme enters the bloodstream. This was effectively prevented by converting the CPG2 from secretory form to intracellular or cell membrane-anchored enzyme, resulting in immune-response reduction [62].

Another example is a few of the pre-existing Cytochrome-P450/Ifosfamide and cyclophosphamide systems [62]. In general, CYP reductase activates all the CYP-dependent activities. Therefore, GDEPT improves the expression of CYP, particularly for the tumorous region, for greater activation of the prodrugs within the cancerous cells only, and showcased a stronger bystander effect. Moreover, Günther *et al.* [65] showed the enhancement in the activity of the bystander effect around the hypoxic region and limited diffusion of the activated drug. They also concluded that only 25% of the CYP administered expressed cells were capable of causing total kill up to 80% (even in a hypoxic environment) [65]. The combination of CYP-cyclophosphamide has successfully entered clinical trials and was found to be safe and well-tolerated [62].

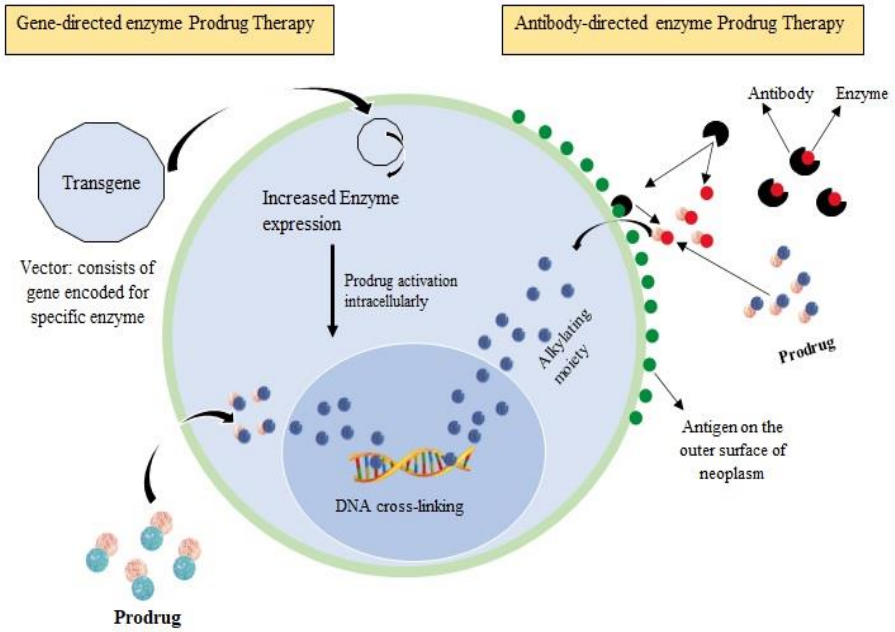

Figure 8. GDEPT vs. ADEPT. GDEPT is an intracellular activation of the prodrug by the overexpression of enzyme using transgene, whereas, in ADEPT, the prodrug is activated by the enzyme release from the antibody. The activation occurred outside the cell to release the active alkylating metabolite. The active alkylating drug has then entered the nucleus through the cytosol.

To further enhance the above gene expression, Jounaidi and Waxman [66] use replication-malfunctioning adenovirus (VDEPT) as a bicistronic carrier with IRES genetic order in combination with CYP2B6 and P450 reductase. CYP-IR-P450 complex undoubtedly enhanced the overall gene delivery as well as the activation of the desired prodrug. Furthermore, genetically modified human macrophages are also introduced by O Kan *et al.* [67]. Here, invasive tumorous properties of macrophages are used as a delivery vector for cytochrome P450 2B6 gene-encoded enzyme, followed by the prodrug administration of

cyclophosphamide. However, these are merely companions to the above VDEPT system. They were proved to display advanced cytotoxicity in combination with VDEPT system only [67]. Several other systems have been used alone or in combination with VDEPT, such as *E. coli* derived enzyme nitro-reductase [68].

In addition, *E. coli* derived enzyme nitro-reductase [68] and *Bacillus licheniformis* derived YfkO nitro-reductase [69] are successfully used with the prodrug di-nitro-benzamide (CB1954) as well as can be immobilized onto the novel gold-coated magnetic nanoparticles as the delivery carrier for a modified enzyme called as magnetic nanoparticles directed enzyme prodrug therapy [69]. Bacteria have been used since 1910, when researchers have found colonized bacterial accumulation in tumor tissues has a positive effect. BDEPT systems are common. The major bacterial carrier used are *E coli* K12, CFT073, HJ1020, *Bifidobacterium*, *Magnetospirillum magneticum*, *E coli.*, *Salmonella*, and DH5α [70]. Figure 8 represents a comparison of GDEPT and ADEPT modes.

3.5.5. Glutathione S-transferase (GST) stimulus for activation of Mustard drugs.

Glutathione is a tripeptide cellular defender for the detoxication of various toxic ions. Chemo drug metabolites are also considered toxic and therefore removed by the G-thione, resulting in multidrug resistance in tumor cells [71]. Also, their detoxification requires two enzymes; Phase I and Phase II. GSTs are typically under Phase II class. GST enzyme is responsible for the secretion of G-thione, unfortunately, proven to be highly expressed within the tumor cells and thus considered a therapeutic target. In fact, there is the expression of around eight isoenzymes of GST, such as α, μ, π, and θ, defined by biochemical properties [72], in different neoplastic tissues. P1-1 GST is elevated in many neoplasms. Lyttle *et al.* created chemo-agents that are suitably activated by the P1-1 GST-rich environment [73]. They later synthesized different GST isomer enzyme selective inhibitors by two-step modifications. First, the C-end changes for supreme variation in catalytic productivity. The second consists of functionalization of sulfhydryl group with alkyl or aryl groups to increase the isozyme specificity at its extreme [72].

Dong *et al.* [74] have stated not one but two different roles of GST π in neoplasm. First, the enzyme wanes the drug efficacy, which causes resistivity. Secondly, it acts as the MAP kinase pathway inhibitor to prevent tumor apoptosis. Since enzyme-prodrug systems have become common, GSTπ induced prodrugs were also introduced to establish controlled activity. For instance, Canfosfamide (TLK286) by Dourado *et al.* [75], i.e., L-γ- Glutamyl-3-(bis[bis(2-chloroethyl) amino-phosphinyl] oxy) ethylsulfonyl-L-alanyl-2-phenyl-[2R]-glycine hydrochloride salt [74] is one of the most prominent G-thione analog prodrug with clinically proven trials. The prodrug is metabolized into glutathione derivative and phosphorodiamidate compound. G-thione prevents the drug resistivity and the latter metabolite act as alkylating agent for apoptosis.

3.5.6. CNS penetrating drugs carrying Nitrogen mustard.

The efficacy of tumor drugs for CNS is limited due to the Blood-brain barrier. Spiromustine (SM) is one of the drugs which is capable of penetrating the CNS effectively. However, it became possible only by combining SM with a lipophilic moiety. One such lipophilic agent is Aziridinyl quinone [76]. Peng *et al.* [77] have prepared several hydantoin derivatives as prominent lipophiles. A combination of spiromustine and hydantoin can act as

excellent CNS penetrating anti-neoplastic agents. Later, many lipophilic agents were used in combination with the mustard drug. Barbituric acid and Vitamin nicotinic acid by Bartzatt *et al.* [78] carrying mustard agent, benzodiazepine, 5-nitroaminobenzophenone by derived mustard agents by Singh *et al.* [30,79,80].

3.5.7. Modern strategies using nitrogen mustard.

Various strategies have been developed to introduce control release of drugs. For instance, chlorambucil is inserted into HPMA vector [poly{N-(2-hydroxypropyl) methacrylamide}] via an ester bond to increase the efficacy of CBL. This is a nanoparticle system with an average diameter of 200nm through a self-assembled macromolecular strategy [81]. With recent development in research, thermosensitive conductive poly-pyrrole microgels have been synthesized for switchable electrochemical sensing of CBL [82]. This thermo-reversible sensor manifests the controlled release of CBL. Use of Single-walled Carbon nanotube (SWCNT) due to chemical and mechanical stability, have high cell uptake, large surface area with different shapes, and thus used intensively to carry biomolecules (proteins, peptides, and nucleic acid) and even drugs. SWCNT can be used in pristine and functionalized forms to deliver drugs [83]. Theranostic prodrugs with multi-organelle attacking are rare but subsequently become very effective. One such is done by Zhuang and co-workers [84]. An esterase enzyme (overexpressed in tumor region) sensitive prodrug tetraphenylethylene functionalized quinolinium-ester-Chlorambucil (TPE-QE-CBL) has been introduced, which allows the selective release of the CBL followed by activation of fluorescence and photosensitization of TPE-QE.

Moreover, it attacks both mitochondria and lysosomes, which results in enhanced antitumor potency. Along with targeted and conventional chemotherapy, metronomic chemotherapy has also become popular. This type of therapy involves the administration of the drug (Cyclophosphamide is commonly used) in a low dose that can remain prolonged and increase drug tolerance [85]. This approach is effective for both solid and hematologic tumors. Chromone synthetic scaffolds have nowadays become popular as they promote the development of anticancer drugs and enhance activity, such as Chromone-Nitrogen mustard derivative [86]. Since cancer cells have an increased level of ROS (Reactive oxygen species) than normal cells, ROS-induced prodrugs have been studied for a while. However, only a few drugs have successfully shown *in vivo* efficacy and selectivity. Fan and co-workers [87] introduced two different ROS-based prodrugs, CWB-20145 and FAN-NM-CH_3, that reduced the size of Triple-negative breast cancer. In 2021, a new class of mustard-based alkylating agents was introduced; bis-3-chloropiperidines (B-CePs) [88].

4. Conclusions

All over the world, millions of people have cancer each year. Therefore, it has become one of the most challenging and requisite fields of medicine. Researchers are still finding better alternatives for greater selectivity, minimal toxicity, and negligible side effects despite existing approaches. This review has emphasized the potency of nitrogen mustard since it has achieved remarkable importance due to its diverse activity performed by a nitrogen atom in the compound. This review has demonstrated at our best every analog of Nitrogen mustard, such as mechlorethamine, chlorambucil alone, and with the combination in different chemotherapeutic strategies. It also comprehensively articulated the compounds that can

reduce the mustard electrophilicity, which reduced their toxicity and thus enhanced their performance. Later, the prodrug has become one of the promising methods due to its extracellular target-region activation; these prodrugs are then used in different systems such as ADEPT and GDEPT. The activation of prodrugs in these systems are fruitfully in-target which established a whole range of different methods of targeting various drugs. Currently, major research focuses on gene therapies for cancer treatments. In drug technology, the synergy of nanotechnology and polymers has been efficiently contributing to novel drugs where the selectivity and cytotoxicity increase with the least deleterious effects.

Funding

This research received no external funding.

Acknowledgments

The authors are thankful to Amity University Uttar Pradesh for providing the necessary facilities to complete this review work.

Conflicts of Interest

The authors declare no conflict of interest.

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