Cytotoxic and genotoxic effects of melphalan in normal and cancerous cells

Tiba Badr Saber ALrifaie

Department of Biology, College of Medicine, University of Tikrit, Iraq

Correspondence Author: Tiba Badr Saber ALrifaie

Received 20 Feb 2023; Accepted 5 Apr 2023; Published 16 Apr 2023

Abstract

Chemotherapy toxicity is a major clinical issue for individuals with advanced cancer. Melphalan is in charge of creating covalent between DNA strands. There exists a correlation between the quantity of intrastrand cross-links created and the drug's in vitro cytotoxicity as well as the patient's response to therapy. These illnesses continue to be a significant public health concern even with the ongoing advancements in the strong therapeutic and pharmacology impact of novel therapy options for cancer patients. Determine the effects of melphalan on both malignant and normal cells, we assessed the cytotoxicity of melphalan derivatives, which revealed increased cytotoxicity. We evaluated the test chemicals' cytotoxic potential against both normal L20B cells and cancerous cells. MEL and its derivatives had dose-dependent cytotoxic effects. The concentration required to 50% block the biological process in vitro was found to be the IC50 value. Chemical structure-biological activity analyses may be based on the results of the conducted study, which also made it possible to choose the molecule with the highest biological activity.

Keywords: cytotoxic, genotoxic, melphalan, amn-3, hela and l20b cell line

Introduction

Even with ongoing medical advancements, cancer is still a serious health issue. It frequently results in mortality and affects individuals of all ages. Numerous potent anti-cancer medications have the capacity to alkylate multiple proteins, RNA, and DNA. The primary alteration that results in anticancer action is DNA alkylation. In the 1942 battle against cancer. The first effective anticancer chemicals were nitrogen mustard-based DNA alkylating agents, which are still widely used as significant medications to treat various cancers. Numerous chemicals with potential medicinal applications have been identified as a consequence of many years of nitrogen mustard study. By decreasing the electrophilicity of mustard agents, safer equivalents can be created through the creation of active compounds. Using this method, clinically effective anti-cancer medications including cyclophosphamide, estramustine, melphalan, mechlorethamine, and chlorambucil were developed. This remarkable class of chemicals functions biologically by binding to DNA, inhibiting DNA replication, and causing cell cycle arrest, which results in cell death. The N7 nitrogen on guanine DNA bases is bound by these alkylating chemicals. Alkylation of DNA happens in two steps. First, a highly reactive and unstable aziridinium cation is formed by the one-step, first order SN2 cyclization of the bis(2-chloroethyl) amine at neutral or alkaline pH. A DNA nucleophile adds nucleophilically to the resultant aziridine cation in the second step, forming a monoalkylation adduct via the SN2 mechanism. These reactions can then be repeated to create a cross-link between two complementary DNA strands by utilizing CH2CH2Cl [1]. Numerous medications and substances that combine to generate reactive electrophiles, such as alkylating compounds, bind to proteins and other macromolecules in the cell to promote the creation of heat shock proteins by attaching covalently to nucleophilic functional groups. Alkylating chemicals also result in additional cytotoxic signals that trigger a heat shock response, including lipid peroxidation, oxidative stress, elevated cellular calcium, and glutathione depletion [2]. The alkylating medication Melphalan (MEL, brand name Alkeran TM) is a member of the nitrogen mustard alkylating agent family. The second part of the 20th century saw the first synthesis of this medication. Nitrogen mustard's phenylalanine derivative is called melphalan [3]. Melphalan's cytotoxic effect inside cells is derived from the cross-linking of DNA between or within structural segments as well as DNA with proteins through the presence of two chlororethyl groups on the molecule. These cross-links cause strand breakage, open ring formation, and nitrogen base loss in the DNA molecule, which interferes with transcription and replication of DNA. Melphalan's capacity to create intra- and interstrand connections makes it a bifunctional alkylating agent [4].

Apoptosis

The intricacies of a certain type of cell death that transpires in various human body cells are now the subject of intense curiosity. The process of programmed cell death (PCD), also known as apoptosis, generally starts fast and affects a limited number of isolated cells instead than all the cells in a particular location. Thus, the gene-regulated process of apoptosis is responsible for morphological alterations. It plays a major role in several autoimmune and neurological illnesses and disorders, in addition to cancer and AIDS. Apoptosis is also triggered by heat, radiation, ROS, hypoxia, and cytotoxic chemotherapeutic drugs, among other detrimental conditions. [5].

Material and methods

Cell culture

Three cell lines were used in this experiment: AMN-3, HeLa, a cancer cell line, and L20B, a normal cell line. The cells were cultivated in tissue culture flasks produced by Falcon USA (Invitrogen, USA), which were supplemented with Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), and 1% antimycotic antibiotic (Invitrogen, USA). The parameters for cell culture were 95% relative humidity, 5% CO, and 37°C. The trypan blue exclusion method was used to measure cell densities, with a final cutoff of 1104 viable cells per milliliter. The 96-well microplates (Falcon, USA) utilized in the investigation had 1103 cells in each well. Within 24 hours of the cell culture, the medication was introduced.

AMN-3 cancer cell line

Mammary adenocarcinoma is adapted for transplantation into cancerous settings. This cell line was generated from female Balb/c mice carrying the AM-3 mammary adenocarcinoma cell line in vivo (AM3).

Hela cancer cell line

He created this line in 1951 from the cervical cancer cells of a woman named Heinrita Lacks and has since been used in thousands of laboratory cell cultures.

$L_{20}B$ normal cell line

This line was derived from mouse L fibroblast cells that express human Poliovirus receptors. The mouse L-cell genome, which is resistant to Poliovirus infection because it does not contain a Poliovirus receptor, has been transformed with Poliovirus-sensitive (human) Hela cell DNA, and L20B cells have been introduced for routine use by World Health Organization (WHO) laboratories.

Cytotoxicity assay

The CCK-8 test was selected to find out how melphalan affected the proliferation of cells. Three test groups were given melphalan twenty-four hours later at amounts that were typically diluted. RMPI 1640 was used in place of the control group's reagent. Each well had a total medium volume of 100 µl. Four hours before to detection, 10 µl of CCK-8 solution was added three days after the medication was introduced. Following that, the absorbency (A450 value) was determined, and the cell growth rates were calculated.

Statistical analysis

The statistical done by program (SPSS) used to evaluate level of ($P \leq 0.05$) ($P \leq 0.01$) and ($P \leq 0.00$) data at a probability

Results

There are noticeable results in the growth of typical AMN-3 cancer cells at 24 hours after treatment with concentrations (800, 400, 200, 100 and 50) of Melphalan, and significant differences were found at the 0.05 level in an average setting of differentiated cells compared to the negative control table (1) and figure (1) Significant differences were also found at the level of $p<0.05$ after 72 hours of disinfectant with all concentration compared to the aloe control.

Table 1: Effect of Melphalan on AMN-3and $L_{20}B$ cell line

Concentration µg mL^{-1}	Mean viability (%) ± SD	
	AMN-3	$L_{20}B$
800	59.01±2.21	63.28±6.9
400	70.12±2.01	67.35±2.8
200	83.24±2.1	81.73±1.90
100	91.04±3.9	89.26±1.88
50	98.32±0.9	97.01±0.91

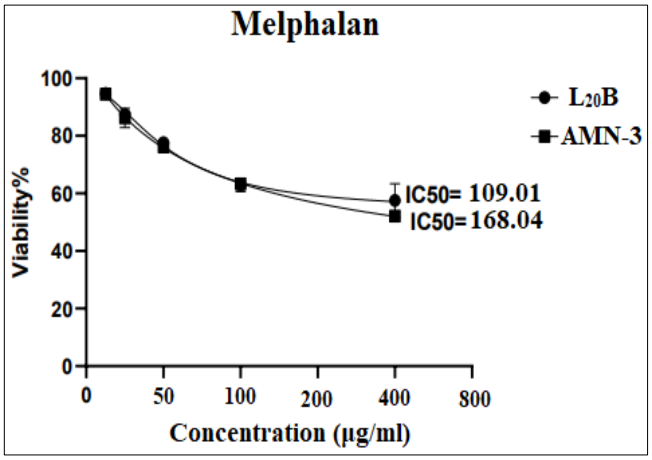

Fig 1: Effect of Melphalan on AMN-3and $L_{20}B$ cell line

Table 2: Effect of Melphalan on Hela and $L_{20}B$ cell line

Concentration µg mL^{-1}	Mean viability (%) ± SD	
	Hela	$L_{20}B$
800	55.6±6.1	56.65±3.01
400	70.23±3.2	71.14±1.9
200	77.9±2.6	78.4±1.5
100	90.24±1.8	91.56±2.88
50	95.36±0.88	96.54±0.9

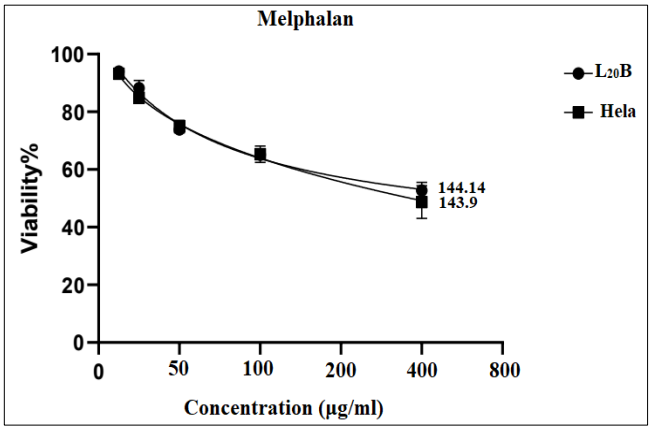

Fig 2: Effect of Melphalan on Hela and $L_{20}B$ cell line

There are few results that significantly affect the growth of cancer cells. Significant differences were observed at the p=0.000 level in recording live cells after 24, 48 and 72 hours of concentrations (800, 400, 200, 100 and 50) of melphalan compared to the negative control table (2) and figure (2).
The results also showed a toxic effect on the growth of HeLa

cells when treated with concentrations (800, 400, 200, 100 and 50) of Melphalan. Small significant differences were observed at the p=0.000 level in the average numbers of live cells after treatment for 72 and 48 hours compared to the negative control. Significant differences were also observed at the $p<0.05$ level in the average numbers of live cells after treatment for 24 hours with the concentration (800, 400, 200), 100 and 50) of Melphalan compared to the negative control table (2) and figure (2).

It was found that the toxic effect was represented by severe cell damage and a change in cell shape.

The results indicate that there is a toxic effect on the growth of cells of the L20B cell line in relation to treatment with Melphalan, as significant differences were found at the $p<0.01$ level in the average numbers of live cells after 24 hours of treatment with concentrations (800, 400, 200, 100 and 50) compared to the negative control. Highly significant differences were also observed at the p=0.000 level in the average numbers of live cells after 48 hours of treatment compared to the negative control. Significant differences were also observed at the $p<0.05$ level in the average numbers of live cells after treatment after 72 hours compared to the negative control (table 1,2) and (figure 1,2).

Manifestations of toxicity were an increase in the frequency of cells undergoing necrosis and apoptosis and cells of unusual shape.

The results indicate that there are clear differences in cell responses based on the type of substance being tested, the cell line, and the duration of treatment for each substance. This indicates the sensitivity of the test to the mentioned factors. It was observed that there were significant differences in the numbers of live cells of the AMN-3 cell line in cultures treated with all tested concentrations of Melphalan for periods of 24, 48 and 72 hours compared with positive and negative control cultures (table 1,2) and (figure 1,2).

This can be explained by two possibilities: the first is the resistance of these cells to the materials used, and the second is the presence of a defect in the preparation of the agents under test. However, the results of the following transactions suggest the first explanation. Significant differences were found in the numbers of live cells of the same passage of the cancer cell line mentioned in the cultures treated with Melphalan after 48 hours of treatment compared to the untreated control. Significant differences were also found after 72 hours (table 1,2) and (figure 1,2) of treatment with all concentrations of Melphalan. Compared with the control plots, this indicates an increase in the effect with increasing concentration at 48 hours of treatment, while differences in concentration do not affect at 72 hours of treatment, meaning that increasing the duration of exposure was the influencing factor.

Treatment of cells from the Hela cancer line showed a similar effect with all concentrations of Melphalan. Highly significant differences were found in the number of live cells in the treated cultures compared to the control cultures after 24, 48 and 72 hours (table 1,2) and (figure 1,2). This indicates the sensitivity of Hela cancer cells to treatment with Melphalan, and this means that there may be an opportunity to benefit from this substance as a chemotherapy in cervical cancer if there is evidence to support this trend from in-vivo studies.

As for the cells of the L20B cell line, it was found that there was an effect of treatment with higher concentrations of Melphalan. Significant differences were observed in the numbers of live cells in cultures treated with all concentrations after 24, 48, and 72 hours (table 1,2) and (figure 1,2).

Discussion

Chemotherapy toxicity is a major clinical issue for individuals with advanced cancer. Melphalan is in charge of creating covalent connections that bind DNA strands together, which stop replication and transcription and ultimately cause the death of cells. There exists a correlation between the quantity of interstrand cross-links created and the drug's in vitro cytotoxicity as well as the patient's reaction to therapy [6]. We evaluated the test chemicals' cytotoxic potential against both normal L20B cells and cancerous cells. MEL and its derivatives have dose-dependent cytotoxic effects. The concentration required to 50% block the biological process in vitro was found to be the IC50 value. When tested against neoplastic cells, the EM-T-MEL derivative had the maximum cytotoxicity, with an IC50 of Concurrently, this substance exhibited a 2.5-fold reduction in cytotoxicity when compared to L20B. The findings from our earlier research [7] were supported by these data, which showed that chemical alterations including the process of esterification involves changing an amino group into an amidine group containing two heteroatoms have strong cytotoxic effects on cancer cells and the similar effects on L20B. The characteristics of the molecule undergo substantial alteration due to esterification and amino group modification.

The literature has also demonstrated that esterification increases cellular penetration and bioavailability [8-11]. Amidine analogs of melphalan have also been demonstrated in other in vitro investigations to decrease the number of breast cancer cells that express estrogen receptors and those that do not [12, 13].

The expression of LAT1, an activating antigen in T cells that is expressed more in T cell leukemia than in normally activated T cells, is changed in nonsolid tumors [14, 15].

Compared with melphalan, we found that the new compounds caused greater differences in the sensitivity of leukemia cells and normal cells. In order for leukemia cells to develop rapidly and continue to multiply, they need lots of nutrients and amino acids. Newer derivatives may offer broader distribution techniques than melphalan due to the lower polarity of the molecule [16].

An ATP-gated ion channel called P2X7R is overexpressed in AML and is widely expressed in malignant cells. Recent studies have shown that opening of P2X7R-activated macropores enhances the intracellular uptake of drugs, such as doxorubicin, a type of chemotherapy. As a result, it has been suggested that P2X7R-induced macropore opening represents a tumor cell-specific drug delivery mechanism [17].

Melphalan, an alkylating drug, also phosphorylates checkpoint kinase 1 (CHK-1) and checkpoint kinase 2 (CHK-2) in MM

cells (RPMI8226 and MM1.S) [18], leading to the formation of an intrastitial DNA junction. - Links that stimulate apoptosis [19]. This elevation in the DNA double-strand break marker (H2AX) is a result of these actions. The original drug and its derivatives caused progressive damage to DNA over time. The EM-T-MEL derivative was the most cytotoxic and produced the greatest amount of DNA damage in all cells examined.

Determining the mechanism by which a cancer cell exposed to the test compounds is destroyed was one of the major scientific problems in this effort. The goal of chemotherapy is to cause apoptosis, a process that is tightly regulated by a number of biochemical pathways involving the expression of several genes. Aberrant apoptosis is one of the primary mechanisms of treatment resistance. An important aspect to consider when evaluating the therapeutic efficacy of a drug is its ability to induce apoptosis.

Apoptosis is the preferred method of cell death because it is a healthy process that does not involve inflammation. Some physical and biochemical changes, such as cell shrinkage, nuclear fragmentation, chromatin condensation, breakdown of cell membrane asymmetry, and formation of small vesicles (ApoBD), separate apoptosis from necrosis [20, 21]. One of the early signs of apoptosis is a change in phosphatidylserine translocation and cell membrane asymmetry.

Improving the effectiveness of therapeutic interventions requires understanding the processes by which cytotoxic drugs suppress cancer cell growth and induce apoptosis. The process known as programmed cell death (PCD) is an energetically demanding, polygenic process that can occur in several ways involving distinct cellular organelles. Intracellular caspases belonging to a broad family of serine proteases play a role in the process of apoptosis. These enzymes play a dual role in apoptosis: during the initiation phase (involving caspases 2, 8, 9, and 10) and the effector phase, which occurs at the end of the process (with caspases 3, 6, and 7). Caspase 9 mainly functions within the intrinsic pathway of apoptosis, where mitochondria play a key role in controlling cell death processes and apoptotic signaling. The intrinsic pathway mediated by receptor proteins is the primary function of caspase 8 [21, 22].

Mitotic catastrophe is a pathway of cell death that occurs primarily due to DNA damage and microtubule destabilizing or stabilizing factors. It is induced by the formation of aneuploid cells and mitotic failure as a result of defective cell cycle checkpoints [23]. Following caspase 2 activation, cytochrome c release, caspase 3 activation, chromatin condensation, mitochondrial membrane permeabilization, and subsequent development of mononuclear giant cells (MONGC) or multinucleated giant cells (MNOC) occur [24-26]. Mitotic failure occurs in a p53-independent manner.

This may indicate that this death pathway is triggered in cancer cells upon incubation with the melphalan analogues under investigation. Subsequent research on new melphalan analogues will attempt to understand the complex process of killing multiple myeloma cells [27].

Conclusions

Anticancer agent mechanisms of action have been clarified thanks to research in cancer biology. It has also served as a foundation for the productive creation of novel medications. The process of creating novel anticancer medication analogs is challenging. the creation of derivatives that possess superior biological properties to the original medicine is made possible by the application of chemical principles, the analysis of chemical structures, and biological activity. The idea that chemically modifying melphalan results in noticeably increased cytotoxic and genotoxic actions has been validated by in vitro research.

References

1. Singh RK, Kumar S, Prasad DN, Bhardwaj TR. Therapeutic journey of nitrogen mustard as alkylating anticancer agents: Historic to future perspectives. Eur. J. Med. Chem. 2018;151:401-433.
2. Liu H, Lightfoot R, Stevens JL. Activation of Heat Shock Factor by Alkylating Agents is Triggered by Glutathione Depletion and Oxidation of Protein Thiols. J. Biol. Chem. 1996;271:4805-4812.
3. Bergel F, Stock JA. Cyto-active Amino-acid and Peptide Derivatives. J. Chem. Soc, 1954, 2409-2417.
4. Falco P, Bringhen S, Avonto I, Gay F, Morabito F, Boccadoro M, *et al*. Melphalan and its role in the management of patients with multiple myeloma. Expert Rev. Anticancer Ther. 2007;7:945-957.
5. Abbas HM. In vitro and in vivo effects of Nigella sativa in normal and cancerous cell. Journal of Advanced Education and Sciences. 2023;3(1):35-41.
6. Van Kan M, Burns KE, Browett P, Helsby NA. A higher throughput assay for quantification of melphalan-induced DNA damage in peripheral blood mononuclear cells. Sci. Rep. 2019;9:18912.
7. Gajek A, Poczta A, Łukawska M, Cecuda-Adamczewska V, Tobiasz J, Marczak A. Chemical modification of melphalan as a key to improving treatment of haematological malignancies. Sci. Rep. 2020;10:4479.
8. Bulumulla C, Kularatne RN, Catchpole T, Takacs A, Christie A, Gilfoyle A, *et al*. Investigating the effect of esterification on retinal pigment epithelial uptake using rhodamine B derivatives. Transl. Vis. Sci. Technol. 2020;9:18.
9. Wang CL, Guo C, Zhou Y, Wang R. In vitro and in vivo characterization of opioid activities of C-terminal esterified endomorphin-2 analogs. Peptides. 2009;30:1697-1704.
10. De Oliveira DP, Moreira TDV, Batista NV, Filho JDDS, Amaral FA, Teixeira MM *et al*. Esterification of trans-aconitic acid improves its anti-inflammatory activity in LPS-induced acute arthritis. Biomed. Pharmacother. 2018;99:87-95.
11. Chvapil M, Kielar F, Liska F, Silhankova A, Brendel K. Synthesis and evaluation of long-acting D-penicillamine derivatives. Connect. Tissue Res. 2005;46:242-250.
12. Bielawska A, Bielawski K, Anchim T. Amidine analogues of melphalan: Synthesis, cytotoxic activity, and DNA binding properties. Arch. Der Pharm. 2007;340:251-257.

13. Bielawski K, Bielawska A, Sosnowska K, Miltyk W, Winnicka K, Pałka J. Novel amidine analogue of melphalan as a specific multifunctional inhibitor of growth and metabolism of human breast cancer cells. Biochem. Pharmacol. 2006;72:320-331.
14. Hayashi K, Jutabha P, Endou H, Sagara H, Anzai N. LAT1 Is a Critical Transporter of Essential Amino Acids for Immune Reactions in Activated Human T Cells. J. Immunol. 2013;191:4080-4085.
15. Zhao Y, Wang L, Pan J. The role of L-type amino acid transporter 1 in human tumors. Intractable Rare Dis Res. 2015;4:165-169.
16. Mynott RL, Wallington-Beddoe CT. Drug and Solute Transporters in Mediating Resistance to Novel Therapeutics in Multiple Myeloma. ACS Pharmacol. Transl. Sci. 2021;4:1050-1065.
17. Pegoraro A, Orioli E, De Marchi E, Salvestrini V, Milani A, Di Virgilio F, *et al*. Differential sensitivity of acute myeloid leukemia cells to daunorubicin depends on P2X7A versus P2X7B receptor expression. Cell Death Dis. 2020;11:876.
18. Lee CK, Wang S, Huang X, Ryder J, Liu B. HDAC inhibition synergistically enhances alkylator-induced DNA damage responses and apoptosis in multiple myeloma cells. Cancer Lett. 2010;296:233-240.
19. Cordelli E, Cinelli S, Lascialfari A, Ranaldi R, Pacchierotti F. Melphalan-induced DNA damage in p53(+/□) and wild type mice analysed by the comet assay. Mutat. Res. 2004;550:133-143.
20. Xu H, He J, Zhang Y, Fan L, Zhao Y, Xu T, *et al*. Synthesis and in vitro evaluation of a hyaluronic acid-quantum dots-melphalan conjugate. Carbohydr. Polym. 2015;121:132-139.
21. Keoni CLI, Brown TL. Inhibition of apoptosis and efficacy of pan caspase inhibitor, Q-VD-OPh, in models of human disease. J. Cell Death. 2015;8:JCD-S23844.
22. Xu X, Lai Y, Hua ZC. Apoptosis and apoptotic body: Disease message and therapeutic target potentials. Biosci. Rep. 2019;39:BSR20180992.
23. Broker LE, Kruyt FAE, Giaccone G. Cell death independent of caspases: A review. Clin. Cancer Res. 2005;11:3155-3162.
24. Vitale I, Manic G, Castedo M, Kroemer G. Caspase 2 in mitotic catastrophe: The terminator of aneuploid and tetraploid cells. Mol. Cell. Oncol. 2017;4:e1299274.
25. ˇCáˇnová K, Rozkydalová L, Vokurková D, Rudolf E. Flubendazole induces mitotic catastrophe and apoptosis in melanoma cells. Toxicol. Vitr. 2018;46:313-322.
26. Mc Gee MM. Targeting the Mitotic Catastrophe Signaling Pathway in Cancer. Mediat. Inflamm. 2015;2015:146282.
27. Erenpreisa J, Kalejs M, Ianzini F, Kosmacek E, Mackey M, Emzinsh D, *et al*. Segregation of genomes in polyploid tumour cells following mitotic catastrophe. Cell Biol. Int. 2005;29:1005-1011.