Covalent Modifications of Melphalan Potentiate its Anticancer Effects

Karan Jani1, Kyle Bashear2, Aldin Husicic3, Joseph Garner3, Kathy Nugent3 and M TinoUnlap4,4*

1UAB Center for Community Outreach Development, University of Alabama at Birmingham, Birmingham, AL 35294, USA
2UAB Science and Technology Honors, University of Alabama at Birmingham, Birmingham, AL 35294, USA
3Department of Clinical and Diagnostic Sciences, University of Alabama at Birmingham, Birmingham, AL 35294, USA
4Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, AL 35294, USA

Abstract

Covalent modification of existing drugs in order to generate alterations that might show increase in efficacy while reducing toxicity has shown great potential in speeding up drug discovery while reducing cost. Melphalan is an antineoplastic agent that works by alkylating, crosslinking, and inducing mutation in DNA which prevent DNA replication and induce apoptosis in malignant cells. Because of its toxic side effects, we proposed and tested the hypothesis that melphalan can be covalently modified to increase its antineoplastic effects. This hypothesis was tested by generating melphalan gluconamine (MG) and melphalan gluconamine methyl ester (CMG) and testing their effects, along with that of melphalan (M), on their ability to attenuate cell proliferation and cell migration and induce apoptosis in MDA-MB-231 breast cancer cells. Treatment of MDA-MB-231 cells with 10 µM of M, MG or CMG for 24 hrs showed that MG and CMG attenuated cell proliferation by 35% ± 2 and 50% ± 10, respectively, while the parent compound did not have any effect of the three compounds, only CMG induced apoptosis and all three inhibited cell migration with CMG showing the greatest effect, inhibiting cell migration by 94% ± 1 compared with 85% ± 2 and 78% ± 1 for M and MG. These studies show that covalent modification of existing drugs is an excellent method of generating analogues which can have greater therapeutic efficacy than the parent drugs.

Introduction

Melphalan, or L-phenylalanine mustard (Figure 1), is a drug that was first synthesized in 1953 when Bergel and Stock substituted the methyl group on nitrogen mustard with phenylalanine [1,2]. After its first use in 1958, melphalan quickly became a leading treatment for patients with multiple myeloma (MM). Later, the Intergroupe Francophone du Myélome (IFM) 9502 trial conducted by Philippe Moreau et al. [3] showed 200 mg/m² melphalan (MEL200) to be less toxic but just as effective as other high-dose treatment options [3], and MEL200 became the standard of care for patients 65 or younger undergoing autologous stem cell transplantation for MM.

The cytotoxic effects of melphalan are due largely to the pair of chloroethyl groups on the molecule which can bind to DNA causing the formation of inter- or intrastrand DNA crosslinks and DNA-protein crosslinks [4,5]. These crosslinks can result in base deletions, strand scission and formation of open rings in the DNA molecule; all of which interfere with both DNA transcription and replication. Since the drug can cause both inter- and intra-strand DNA crosslinks, it is classified as a bifunctional alkylating agent. The alkylating effects of the drug are not cell cycle dependent but are related to its concentration and the period of cellular exposure [1].

In addition to its alkylating effects, a study done by Patricia Gomez-Bougie et al. (6) showed that melphalan also causes severe downregulation of antiapoptotic proteins Mcl-1L, Bcl-xL and BimEL while having lesser effects on proapoptotic proteins [6]. The drug cleaves the proteins which results in their down regulation. Furthermore, caspase cleavage of antiapoptotic proteins caused by melphalan induces the generation of proapoptotic cleaved forms [6]. These effects, in combination, produce a greater probability that proapoptotic units will achieve their function and push the cell toward apoptosis.

Because of the length of time and cost that it takes to move a drug from the bench to the market, researchers have explored other options including making improvements of drugs that are already in use [7]. One of the methods was the covalent binding of small molecules or functional groups to an existing drug. Modifications of this nature have the potential to improve the efficacy and duration of the drug, and even possibly reducing its side effects. The numbers of different covalent modifications that can be performed on a single drug are numerous but the number and types of changes any one of these modifications could have on the properties of the drug are even greater. One modification that has seen extensive use in recent years is “PEGylation.” This is the process by which polymers of polyethylene glycol (PEG) are attached to a drug molecule [8,9]. Addition of PEG has been tested mostly on protein and peptide based drugs. Many members of this family of drugs are not very useful in therapy because they have a poor circulation time and are easily degraded, cleared or neutralized in the body. However, various studies have shown that covalently binding PEG to one of these

![Figure 1: The structure of melphalan shows the carboxyl group and amino group which were modified. The amino group was modified by replacing one of the hydrogen with glucose to generate melphalan gluconamine. The carboxyl group, on melphalan gluconamine, was modified by replacing the H with a methyl group to generate melphalan gluconamine methyl ester.](http://dx.doi.org/10.4172/scientificreports.545)
drugs can overcome its deficiencies and produce a safer and more effective drug [9,10].

Many drugs have since been selected and modified in a number of ways in order to improve their efficacies. In 2007, a research team from Poland tested the anti-cancer effects of 5-amidine analogues of melphalan and compared the effects to that of the parent drug. Each drug was used for the treatment of cultures of MCF-7 and MB-231 breast cancer cells. The data showed that every one of the analogues had greater anti-cancer effects on both cell types than unaltered melphalan [11]. Moreover, there were distinct differences between the levels of effectiveness between the analogues allowing them to be easily ranked from most effective to least effective. This order of effectiveness was the same for both cell types [11].

Therefore, based on these findings that covalent modifications of drugs, including Melphalan, increase their efficacies against their intended indications we proposed and tested the hypothesis that covalent modifications of melphalan by glycoconjugation with or without esterification will increase its efficacy against breast cancer. This hypothesis was tested in MDA-MB-231 breast cancer cells by examining the effect of melphalan and its modifications on: 1) cell proliferation, 2) apoptosis, and 3) cell migration.

Materials and Methods

Cell culture

MDA-MB-231 were obtained from ATCC and grown in 50mL flasks using DMEM-reduced serum (Hyclone) supplemented with 5% Fetal Clone III (F(III)) and 1% Pen-Strep. The cells were passaged twice a week. Culture methods and conditions used were as previously described [12].

Cell proliferation assay

MDA-MB-231 breast cancer cells were plated in a 12-welled plate at approximately 50,000 cells per well for 24 hrs. The cells were treated with 10 or 100 µM of melphalan (M) or one of its covalent modifications, melphalan gluconamine (MG) and melphalan gluconamine methyl ester (CMG), followed by aspiration of media. Cells were detached in 250 µl of trypsin, neutralized with equal volume of growth media and counted using a hemocytometer [13]. Cells counts were compared between treated and non-treated samples using the Student’s t-Test. n=3; **p ≤ 0.001; *p ≤ 0.05.

Apoptosis assay

MDA-MB-231 breast cancer cells were plated in 100mm plates at approximately 500,000 cells per well for 24 hrs. Cells were treated with 10 or 100 µM of melphalan (M), or one of its covalent modifications, melphalan gluconamine (MG) or melphalan-gluconamine-methyl ester (CMG), followed by aspiration of the pellets. Supernatant was aspirated and the pellets were resuspended in 5 ml of 1XPBS. The cells were pelleted as previously described and used for apoptosis assay using Life Technologies Caspase-3/7 Colorimetric Assay [14]. Brieﬂy, cells were lysed in 50µl of lysis buffer, centrifuged at 14,000g for 5 minutes and the supernatant was used for protein concentration assay [15]. Equal aliquots, 100µg, of each protein was used for caspase-3/7 activity assay using 4mM of the DEVD-pDNA substrate in a total volume of 100µl in a 96 well plate. The plate was incubated for 2 hours at 37°C and the samples were read at 405 nm using a microplate reader. Absorbance intensities were corrected by subtracting the background and compared between treated and non-treated samples using the Student’s t-Test. n=3; **p ≤ 0.001; *p ≤ 0.05.

Cell migration assay

Agarose plates, 1%, were made using HycloneMEM-reduced serum in 60 mm Petri dishes. The plates were allowed to polymerize and 3 parallel rectangular wells of equal dimension and 0.5 cm apart were made in the gel using a sterilized scalpel. MDA-MB-231 cells were detached by trypsinization, neutralized and aliquoted into the outside wells in a total of 200µl at a density of 100 cells per microliter. Each plate contained cells that were either not treated or treated with M, MG, or CMG at 10 µM for 24 hrs. The plates were incubated at 37°C in a humidified chamber for 24 hrs and the number of cells that had migrated out of the outside wells toward the center well were counted and compared between treated and non-treated samples using the Student’s t-Test. n=3; **p ≤ 0.001.

Results and Discussion

Cell proliferation assay

Our results (Figure 2) demonstrate that unmodified melphalan, at 10 µM and 24 hrs, did not have any significant effects on the proliferation of MDA-MB-231 breast cancer cells. Covalent modification of melphalan by glycoconjugation with or without esterification to generate melphalan gluconamine and melphalan gluconamine methyl ester, respectively, potentiated the ability of melphalan to attenuate the proliferation of this particular breast cancer cell line. The effect of melphalan on the proliferation rate of this cell line was significant (p<0.05) when the 24 hr treatment was increased to 100 µM (Figure 3) but the effect of melphalan was still much less than that of its two modifications, melphalan gluconamine and melphalan gluconamine methyl ester which both showed significance at the
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**Conclusion and Significance**

Our studies demonstrate that covalent modifications of melphalan by glycoconjugation with or without esterification potentiate its anticancer effects against MDA-MB-231 breast cancer cells with melphalan glucosamine methyl ester demonstrating the greatest anti-proliferative, pro-apoptotic, and anti-cell migrating effects, even at the low concentration of 10 µM. The parent compound, melphalan, did not inhibit the proliferation nor induce apoptosis in MDA-MB-231 cells at 10 µM but did inhibit cell proliferation only at 100 µM but even at this high concentration melphalan still didn’t induce apoptosis. The findings of this study demonstrate that an FDA approved drug can be covalently modified to generate altered forms of the drug which can increase the efficacy of the parent compound.

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**References**


