

Chlorambucil-Asparagine, a Novel Chemotherapeutic Agent

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Abstract: As cancer disease has become a significant health issue all over the world, it is important to increase the effectiveness of anti cancer drugs and reduce their adverse side effects. A number of anti cancer drugs have been discovered so far, one of which is known as Chlorambucil. It is a member of Alkylating drugs that attack DNA of cancerous cells and kill them. Great numbers of conjugates have been designed in order to make Chlorambucil more effective and less harmful. A couple of reports have suggested that accumulation and uptake of Polyamine compounds and amino acid transporters increase in cancer tissues. Therefore, we connected an amine amino acid (Asparagine) to Chlorambucil in order to create a novel and efficient anti cancer conjugate. After purifying our product and performing some quality control tests, its anti cancer effects on HT1080 cell line were evaluated. MTT, apoptosis, necrosis and abnormality tests were conducted to check its toxic properties. Finally, it was evaluated if Chlorambucil affects blood hemolysis rate and blood clotting factors or not. It was observed that not only dose Chlorambucil-Asparagine conjugate has anti cancer property, but also it is capable of killing the half of the cancerous cells in lower concentrations compared to Chlorambucil. So, using lower dose of a drug can help reducing its undesirable side effects. Besides, the new drug has no effect on normal growth of Sprague Dawley rats. Also, it is not caused blood hemolysis and does not change the clotting factors. Moreover, it is determined that it can kill cells by mechanism of inducing apoptosis. In conclusion, a new, more efficient, cancer targeted version of an anti cancer drug was synthesized successfully in this study. The results indicate that Chlorambucil-Asparagine conjugate seems to have a bright future for further studies and treatment of cancer.

Key words: Cancer • Chemotherapy • Anti Cancer Drugs • Chlorambucil • Targeted Drug Delivery And Asparagine

INTRODUCTION

Today cancer is identified as one of the biggest health issues all over the globe. After cardiovascular diseases, cancer is the main causes of death and in 2013 it is expected that more than 500 thousand people will die of cancer in America [1]. Cancer treatment methods mainly include surgery, radiation, gene therapy and chemotherapy. Chemotherapy is a general word for any

kinds of medical treatment using drugs and chemical agents towards killing cancerous cells. A number of chemotherapeutic drugs has been designed and synthesized so far and, in despite of their side effects and medical limitations, they have been highly effective in cancer treatment [2].

Anti cancer drugs can be classified into 5 groups of Alkylating agents, Anti-metabolites, Mitotic inhibitors, Topoisomerase inhibitors and Anti-tumor antibiotics

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[2, 3]. Most of these drugs work by affecting the DNA of cells. Alkylating agents are one of the most effective groups which form cross-linkages on DNA stands, so inhibit the semiconservative replication and transcription [4]. They include 5 main classes; Nitrogen mustards, Aziridines, Alkyl sulfonates, Nitrosoureas and drugs containing platinum. Discovery of Nitrogen mustards can be considered as a starting point of cancer modern treatment which are effective and have low toxicity [5]. One of the most common subclasses of Nitrogen mustards is Chlorambucil (4-[bis (chloroethyl) amino] benzenebutanoic acid) abbreviated to CLB in this study. Its molecular weight and chemical formula are 304.21g/mol and $C_{14}H_{19}Cl_2NO_2$ respectively. CLB has been used since 1961 as an anti cancer drug and immunosuppressive agent. It is known as Leukeran commercially and is available in form of tablets for oral administrations [4, 6]. Its chemical structure is shown on Figure 1.



Fig. 1: The chemical structure of Chlorambucil

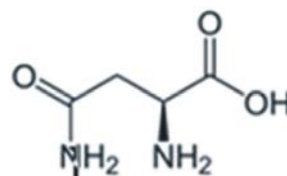


Fig. 2: The chemical structure of Asparagine

As mentioned above, these drugs have their limitations and one of which is lack of selectivity that leads to having adverse side effects and restricted effectiveness [7]. In order to solve this problem, an approach titled “targeted drug delivery” was introduced. The method can be beneficial in several ways such as decreasing drug toxicity, enhancing efficiency and using lower doses [8]. Tumor-specific delivery of therapeutic agents to tumor site requires a carrier capable of connecting to tumor-specific cell markers [9]. Although there are some difficulties in designing carriers such as low solubility of drugs, using nanoparticles can be highly successful in overcoming this problem [10]. Nanoparticles like dendrimers, micelles, nanospheres, nanocapsules, nanotubes and liposomes with their unique structures can be used for targeted drug delivery. Besides, other kinds of carriers can be designed depending of tumor characteristics. These carriers include antibodies, peptides, proteins (Lectin and albumin), amino acids, hormones, mono-, oligo, poly-saccharides, folate and lactose [11].

In this research, it was attempted to find a proper carrier for Chlorambucil. Several conjugated derivatives of this drug have been synthesized and their anticancer properties have been approved in previous studies. For instance, in 1996 regarding to limitations of using chemotherapeutic drugs for brain tumors due to their low uptake in central nervous system, Halmos *et al.* synthesized CLB-glucose conjugate, so increased the uptake rate and efficiency of CLB [12]. In 1998, two separated studies were performed; in which two conjugated derivatives of CLB were synthesized

(CLB-Albumin and CLB-Transferrin). The results show that connecting carboxyl end of these proteins to the drug can increase its anticancer property compared to non-conjugated for mKratz *et al.* [13] and Beyer *et al.* [14]. In 2010, Gupta *et al.* made CLB-Estradiol hybrids and proved their enhanced antitumor characteristics [15]. In the same year, Descoteaux *et al.* [16] synthesized D- and L-Tyrosine-CLB and evaluated its anticancer nature on breast cancer cells. The result indicates that anticancer property of the new drug was increased compared to CLB [16].

In this study for the first time we have connected Chlorambucil to Asparagine amino acid. The idea of using Asparagine comes from two studies carried out by McGivan *et al.* [17] and Russel *et al.* [18]. The results of the former study show that generally amino acid carriers are essential for proliferation and growth of cancer cells. Also, findings of the latter study indicate that Polyamines are accumulated in cancer tissues and their concentrations rise in the fluids of cancer patients’ body. Therefore, it can be concluded that amino acids containing amine groups in their structures can be a suitable carrier for anticancer drugs. Not only do these kinds of amino acids give the drug “selectivity features”, but also they enter the cells more easily with the help of particular delivery systems. Asparagine (2-amino-3-carbamyl propanoic acid) is widely used in drug production and is a nonessential amino acid for the body [19]. Its chemical structure can be seen in Figure 2.

To sum up, the purpose of this research is to synthesize a new version of Chlorambucil with fewer side effects, targeted delivery property, anticancer and cytotoxic effects on tumor cells using Asparagine amino acid.

MATERIALS AND METHODS

Material Preparation: Chlorambucil, ADH (Adipic Acid Dihydrazide), Asparagine amino acid, Sulfo-NHS (*N*-Hydroxysulfosuccinimide Sodium Salt), EDC (1-ethyl-3-(3-dimethylamino propyl) carbodiimide), NaHCO₃, NaOH, HCL, Na₃C₆H₅O₇, Trypan Blue stain and MTT powder were purchased from Sigma-Aldrich company. Besides, PBS (Phosphate Buffered Saline), DMSO (Dimethylsulfoxide), Chloroform, Methanol, Ethanol and FBS (Fetal Bovine Serum albumine) were obtained from Merck Co., RPMI culture medium was also obtained from Biochrom Co.

Synthesis of Chlorambucil-Asparagines conjugate:

The mixture of 5mg Chlorambucil and 5mL PBS solvent (1X) was decanted into a container and placed on a magnetic mixer. Then, 2mL DMSO (To accelerate the solubility process and increase the reaction rate), 10mg EDC (To activate the carboxyl groups) and 2mg Sulfo-NHS (To remove water and enhance the reaction efficiency) were added to it. After a while, 2.9mg ADH (As a linker) was also added to the mixture. It took 5 minutes to complete the mixing process. In a separate container, 5mg Asparagine amino acid, 10mg EDC and 2mg Sulfo-NHS were mixed and placed in room temperature for 5 minutes. Finally, the mixture of the second container was poured into the first one slowly and the final time to complete the synthesis process was 2hours.

Purification and Quality Control: Gel Filtration Chromatography and Thin Layer Chromatography (TLC) techniques were used in order to control and purify the synthesized product. TLC solvent was a mixture of 3mL chloroform and 7mL methanol. Additionally, the spots included the following compounds: Asparagine, ADH, Sulfo-NHS, EDC, Chlorambucil and the synthesized product. The reaction was performed in 25°C for 20min. As all of the spots were colorless, UV radiation (With wavelength range of 220-280 nanometer) was used to make them visible and RF index was calculated for each of the compounds. Finally, an amount of the new drug was turned into powder using Freeze-dryer machine for greater stability.

Liquid Chromatography–Mass Spectrometry: In order to investigate the molecular structure of the new drug, LC/MS technique was used. The machine was manufactured by Agilent Technology (HP) Company.

MTT Assay: In this research, cellular studies were performed on HT1080 cell line and, the culture medium was RPMI (Includes NaHCO₃, mixture of Penicillin and streptomycin antibiotics, HCL, NaOH, Hepes buffer, glutamine and FBS). For MTT assay, particular amounts of cell and its medium were added to 36 wells of a 96-well plate. The new drug (CLB-Asn) was added to 12 wells with the four dosages of 20, 60, 120 and 240μL (Each doses was repeated 3 times). The old drug (CLB) was added to the other 12 wells with the same doses in order to make a comparison. In the next 3 wells, no drug was added (Control group). Finally, the three last wells were assigned to positive control in which PHA (Cell proliferation simulator) was added. After the MTT assay processes, O.D of the samples were read in 570nm using ELISA Reader.

Abnormal Toxicity Test: 40 young Sprague Dawley rats with the average weight of 90±20g were chosen for this study. They were classified into 4 groups and 10 rats were placed in each one (5 males and 5 females). 200 and 230μL placebo were injected into the female and male rats respectively in the first group which was considered as the control. 70 and 80μL Chlorambucil with the dosage of 120μg were injected into female and male rats in the second group. Female and male rats in the next group were received injection of 140 and 150μL Chlorambucil-Asparagines conjugate with the dosage of 60μg. Finally, 200 and 230μL of the new drug with the dosage of 120μg was injected into females and males in the last group. It should be mentioned that all the injection was IP and the measured toxicity was Acute which was performed according to Committee of Proprietors of Medicinal Products (CPMP) and International Committee for Harmonization of Biological compounds (ICHBC) instructions. These animals were weighted in the first, 7th and 14th days of the study and their weight differences were analyzed via SPSS software. Besides, they were checked for any kinds of toxic effects on their skin and hair after each injection. After the period of 14 days, animals were killed using cervical dislocation method and some of their organs such as liver, spleen and mesenteric ganglia were taken for histological studies.

TNF-α Release Test: This test was performed using the RayBio® Human TNF-alpha ELISA kit. HT1080 cell line was incubated with the exposure of 120 and 240μM CLB-Asn conjugate for 24hours. After centrifugation, the supernatant of each tube was evaluated for release of TNF-α (O.D of samples

were read in 450nm via Microplate Reader). It should be mentioned that the test was repeated 3 times and the negative control was 16040-RPMI.

Apoptosis Test: Apoptosis and necrosis cells were recognized by Annexin V-PI coloring (PI: Propidium Iodide). Then, the percentage of these cells compared to control group (Cells exposed by 60 and 100µg/mL Chlorambucil) was determined by Flow Cytometric method via recommended protocol (FACS calibur,BD). Wavelengths of excitation and emission were 488nm and 518nm respectively. This technique can characterize differentiate between the two mechanisms of cell death (Apoptosis and necrosis).

Blood Cell Hemolysis Test: Blood samples were taken from healthy candidates who were not taken any drugs. Platelet-free plasma of blood was obtained using centrifuge in 1000 rpm for 5min. Then, blood samples were incubated in 37°C with Chlorambucil (Solvent in PBS/DMSO) and Chlorambucil-Asparagines (Solvent in PBS/DMSO) for 6 hours. After centrifugation, supernatant absorption was read in 413nm by a spectrometer. To compare the results, there was a positive control (PBS solvent in 0.1% v/v Triton-X100).

Evaluation of Blood Clotting Factors: In order to evaluate changes in blood clotting factors, 2mL blood was taken from a candidate and then exposed to Chlorambucil-Asparagines. Finally, clotting time was compared to control group (Healthy blood).

Table 1: RF index of the spots

Material	Asn	CBL	CBL-Asn	Sulfo-NHS	ADH	EDC
Rf	0.90	0.32	0.63	0.49	0.82	0.88

Table 2: Conditions and characteristics of mass spectrometry

Parameter	Step 1	Step 2	Step 3	Step 4	Step 5
Initial Temperature (°C)	50
Initial Time (min)	0
Program Rate (°C/min)	80
Final Temperature (°C)	350
Final Time (min)	5

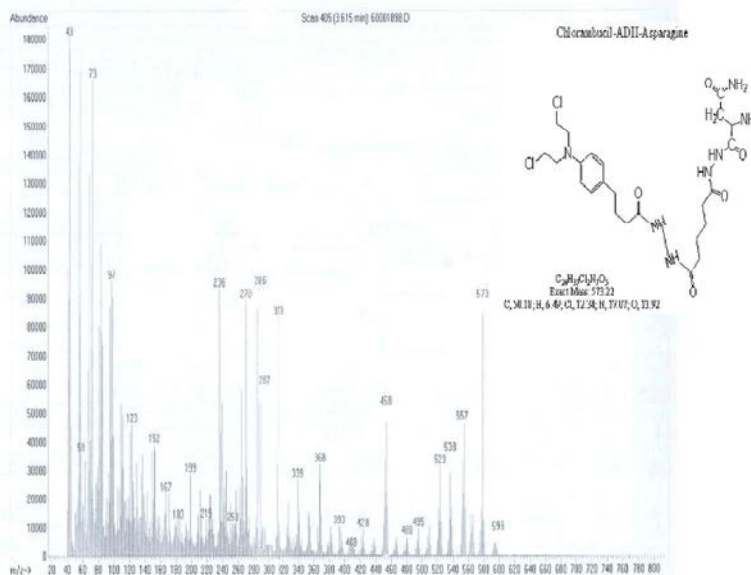
Instrument Specifications:

- Manufacturer Company: Agilent Technology (HP)
- 1.MS Model: 5973 Network Mass Selective Detector
- 2.Ion source: Electron Impact (EI) 70 eV
- 3.Ion source temperature: 230°C
- 4.Analyzer: Quadrupole
- 5.Analyzer temperature: 150°C

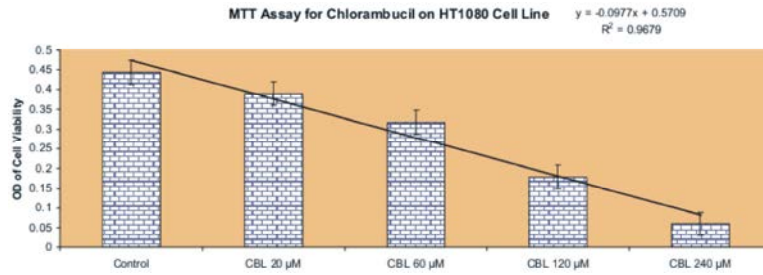
RESULTS AND DISCUSSION

Chromatography: RF index of all the spots are shown on Table 1. As it can be seen, the RF number of CLB-Asn was not similar to the RF of other spots (The primary substances of the reaction) which confirms that CLB-Asn was synthesized correctly.

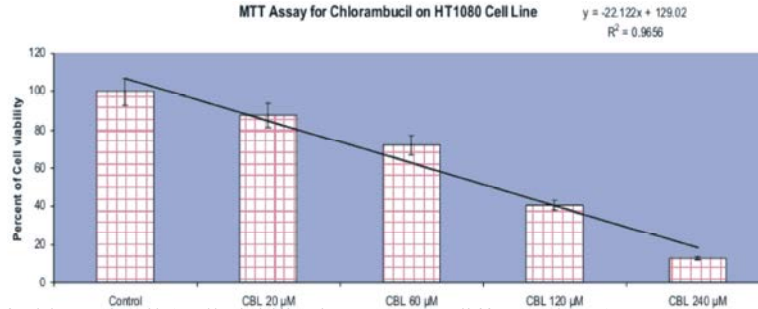
Mass Spectroscopy: Graph 1 and Table 2 show the mass spectrum of CLB-Asn and conditions and characteristics of mass spectrometry respectively. The obtained chemical structure of the conjugate is in accordance with our estimate. Also, the molecular weight is calculated to be 573.22g via this technique.



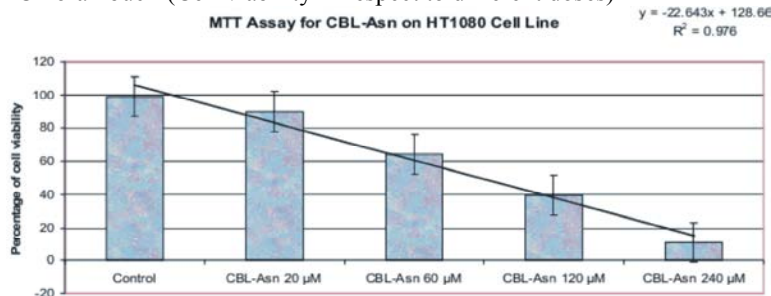
Graph 1: Mass spectrum of CLB-Asn



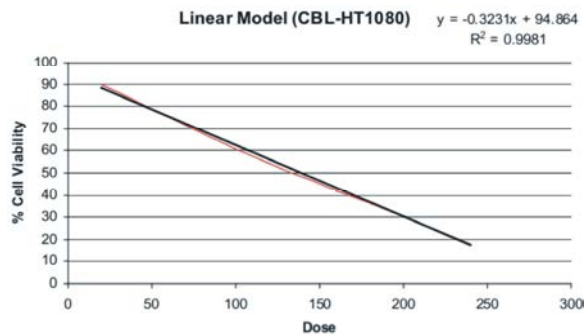
Graph 2: MTT assay of Chlorambucil (O.D in respect to different doses)



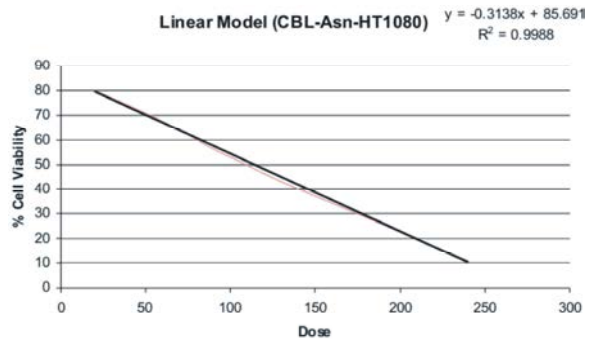
Graph 3: MTT assay of Chlorambucil (Cell viability in respect to different doses)



Graph 4: MTT assay of Chlorambucil-Asn



Graph 5: Linear model chart of CLB



Graph 6: Linear model chart of CLB-Asn

MTT Assay: The results of MTT assay for Chlorambucil in concentration of 20, 60, 120 and 240µM are shown on Graphs 2 and 3. The first graph shows the O.D of samples in respect to these concentrations and the cell viability of the samples is shown in Graph 3. Graph 4 indicates the result of MTT assay for Chlorambucil-Asn in the same concentration. As it is obvious, increasing the concentration of the both drugs leads to decreasing cell

viability. Significant statistical differences can be seen between control and 60, 120 and 240µM of CLB and CLB-Asn ($p < 0.001$). Consequently, the anti cancer property of CLB is maintained in CLB-Asn conjugate.

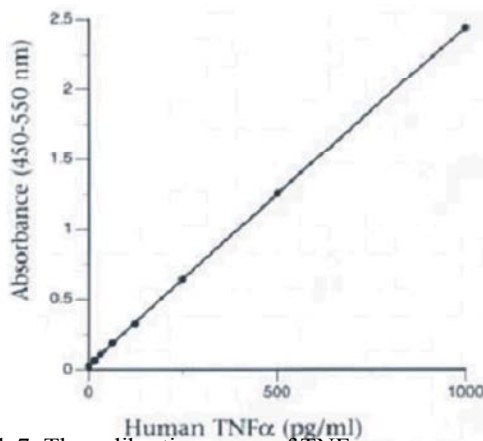
In order to calculate the EC_{50} of these drugs, linear model chart was used (Graphs 5 and 6). EC_{50} of CLB was 138.854µM (calculated by its equation: $y = -0.3231x + 94.864$) and EC_{50} of CBL-Asn was 81.8702µM (Calculated by its

Table 3: Female rat body weights (Mean ± SD)

Group	Day 1	Day 7	Day 14
I	90.6±4.72	135±5.03	158.8±4.44
II	90.40±5.64	130.6±3.21	135.4±8.38
III	90.4±4.04	1.39.8±9.71	148.6±5.77
IV	90.60±5.64	134.8±8.32	137.8±3.42

Table 4: Male rat body weights (Mean ± SD)

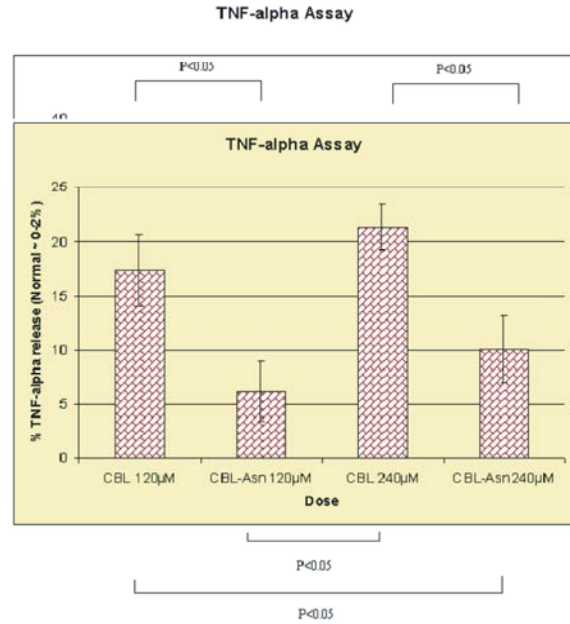
Group	Day 1	Day 7	Day 14
I	100.6±2.28	156.2±7.28	203.2±14.43
II	100.2±3.27	140.4±3.78	155.8±16.5
III	100.6±2.61	153±4.71	192.2±7.76
IV	100.4±3.13	145±6.4	178±8.86



Graph 7: The calibration curve of TNF-α

equation: $y = -0.3138x + 85.691$). Comparing the numbers, it can be concluded that the new drug kills 50% of cancerous cells in the lower concentration compared to CLB.

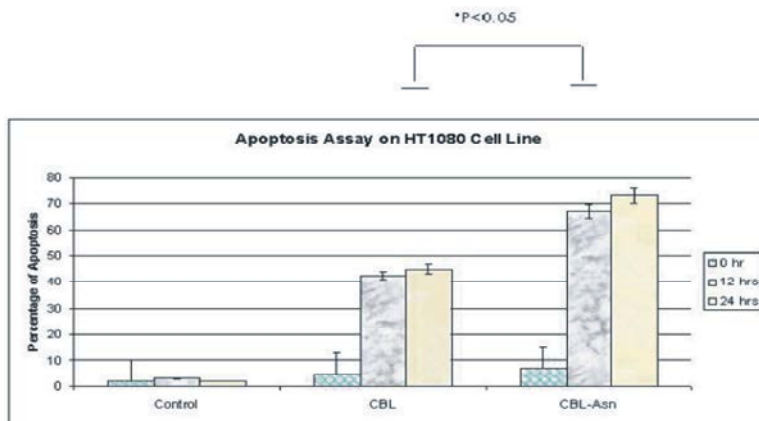
Abnormal Toxicity Test: Tables 3 and 4 show the Mean ± SD of changes in female and male rat body weights respectively. The growing trend of animal weights was considered as normal in the first two weeks which



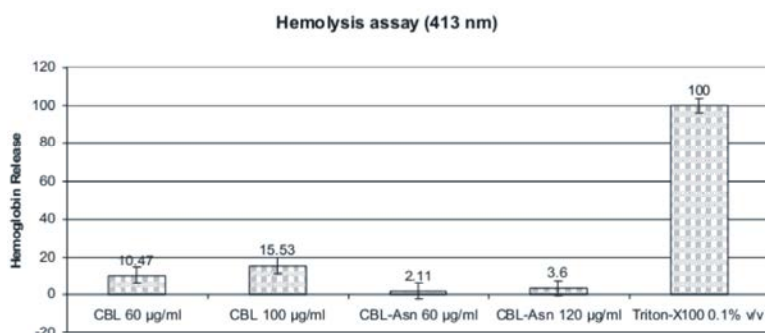
Graph 8: Result of TNF-α release test

indicates the fact that CBL and CBL-Asn had no effect on animal growth weights. Besides, according to these data and our observations, there was no macroscopic harm to animals after injection of CBL-Asn. However, CBL injection caused death of two female and one male rat.

TNF-α Release Test: Graph 7 shows the calibration curve of TNF-α and Graph 8 shows the result of TNF-α release test. In this graph, percentages of the release are compared in two concentrations of CLB and CLB-Asn (120 and 240µM). As it is shown, there are significant differences between the two drugs in the both concentrations ($P < 0.05$). So, it can be concluded that necrosis property of CLB is higher than CLB-Asn in a constant concentration.



Graph 9: The result of the apoptosis test of CLB and CLB-Asn compared to control group



Graph 10: Results of hemolysis test in different doses of CLB and CLB-Asn

Apoptosis Test: Graph 9 shows the results of the apoptosis test of CLB and CLB-Asn compared to control group in the initial (0), 12 and 24 hours. Concentrations of the drugs were chosen to be the highest one (240µM) as the apoptosis phenomenon is not observable in low concentrations. The results show that there was a significant difference in percentage of apoptosis between 0 and 24 hours ($P < 0.05$) and apoptosis property of CLB-Asn was higher than CLB in the constant concentration. By comparing the necrosis and apoptosis graphs, it is determined that the cell death mechanism of CLB-Asn is mainly inducing apoptosis.

Blood Cell Hemolysis Test: Graph 10 shows the results of hemolysis test in different concentrations of CLB and CLB-Asn. The control group (Triton-X100 in PBS) was considered to release 100% hemoglobin. According to the results, CLB could slightly release hemoglobin in 60 and 100µM. The maximum percentage was only 15.53 in the highest concentration (100µM) compared to control group. However, neither 60µM of CLB-Asn nor 120µM of it had any significant blood hemolysis.

Blood Clotting Factors: According to the result of this study, as the blood was exposed to CLB-Asn, its clotting time was not different from the control group (Healthy blood) which confirms that this drug has no effect on blood clotting factors.

CONCLUSION

Since cancer incidence is rising rapidly in the world, synthesis and designing more effective anti-cancer drugs seem essential. In this study, it was attempted to specify one of the oldest anti cancer drug named Chlorambucil using drug targeted delivery method. Previous studies were proved that Polyamines accumulate in cancer and highly metabolic tissues by polyamine transmission

system. So, we choose an amino acid containing amine group in its structure (Asparagine) to connect to Chlorambucil. Consequently, our synthesized product was a polyamine compound (Both Asparagine amino acid and ADH linker contain amine groups) which has the ability to accumulate in cancerous cells. Besides, results obtained from toxicity tests indicate that CLB-Asn keeps the anti cancer property of CLB. CLB has 2 Chlorine atoms which makes it be lethal to cancer. In fact these atoms attack DNA of cells and destroy them by Alkylating process. Therefore, to protect this property, Asparagine amino acid was connected to CLB from the Carboxyl end. Moreover, our synthesized conjugate has lower EC50 compare to CLB which means that it is more toxic in the lower concentrations. As a result, it can be used clinically in the lower doses, so has less adverse side effects for cancer patients.

Additionally, results obtained from TNF- α and apoptosis tests show that cell death mechanism of CLB-Asn is mainly apoptosis induction. This drug has no effect on normal growth of rat weights, blood hemolysis and blood clotting factors.

Although these results are promising, further researches to continue and complete this study are necessary. It is suggested that CLB-Asn conjugate's effects on other cell lines should be evaluated, particularly the ones which needs Asparagine in their culture medium for growth. Besides, *in vivo* studies are also important to obtain more accurate perception of its any possible effects on animal models.

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