

Bicelin (B-Cell Lymphoma 2 Inhibitor) a Highly Safe DNA-based Anti-Cancer Drug Showed no in vivo Cytopenia, Nephrotoxicity and Hepatotoxicity

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ABSTRACT

Background: Non-Hodgkin's lymphoma is the seventh most common cancer in clinic. Lymphoma could be treated with newly developed anti-apoptotic small molecule inhibitors of bcl-2 such as ABT-199 or venetoclax. However most small molecules present numerous side effects and cancer becomes resistance to them rapidly. PNT100 is a DNA-based bcl-2 inhibitor that has shown great efficacy and safety in treating non-Hodgkin's lymphoma tumors. However, liposomal carrier (smarticles) that was used to deliver PNT100 eventually failed to produce robust efficacy in the latest clinical trial conducted by ProNai Therapeutics (now called Sierra Technology). The high cost of liposomal carriers, makes this targeted drug less affordable. In addition, the liposomal components may also present some minor side effects as reported in pilot phase II trial. In this study, we eliminated liposome and used a specific Epigenic modification to deliver this 24 bp oligonucleotides (PNT100) without using additional chemicals and named it Bicelin.

Methods: In this study, the safety of Bicelin was determined by evaluating the cytopenia, nephrotoxicity and hepatotoxicity effects of this drug in healthy animal model (Rats). The rats in experimental group were received Bicelin (20 mg/kg/day) for 5 days a week. The treatment was continued for 3 consecutive weeks. The blood and urine samples were collected for evaluation and the rats were then sacrificed. The kidneys and liver tissues were fixed in formalin 10% to perform histological investigation using H&E staining.

Results: The in vitro and in vivo results clearly demonstrate that Bicelin is target specific and highly safe. We observed no cytopenia when blood tests were performed after 15 consecutive injection of Bicelin; Urine analysis revealed no significant differences between experimental and control group and there was no nephrotoxicity or hepatotoxicity when kidney and liver tissues were examined.

Conclusion: Based on our in vitro and in vivo safety studies, our bcl2 inhibitor, Bicelin is much safer and about 10-fold more effective than its liposomal form (PNT2258). Considering preclinical, phase I and II studies of PNT2258, Bicelin is expected to be very safe and effective in clinic.

Abbreviations: PNT: ProNai Therapeutics; DLCL: Diffuse Large Cell Lymphoma; IC50: (half-maximal) Inhibitory Concentration

Keywords: Bicelin; B-cell lymphoma 2 (BCL-2); Nephrotoxicity; Hepatotoxicity

BACKGROUND

Apoptosis is a regulated programmed cell death that is triggered in defected cells. This selective cell suicide plays an essential role in numerous physiological and pathological processes [1]. There are two apoptotic pathways—the extrinsic pathway (activated by ligand engagement of cell surface death receptors) and the intrinsic (mitochondrial) pathway. The BCL-2 family of proteins regulate activation of the intrinsic apoptotic pathway in response to cellular

stresses such as DNA damage, g-irradiation, oncogene activation etc.

BCL-2, was first identified through chromosomal mapping in follicular lymphoma where constitutive BCL-2 expression is driven from the immunoglobulin locus by the translocation [2-4]. BCL-2 facilitate oncogenesis through cell death resistance [5,6]. Its involvement in Follicular Lymphoma (FL), has a central role in the inhibition of apoptosis. The translocation causes constitutive

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overexpression of BCL2 by juxtaposing it to immunoglobulin heavy chain gene enhancer elements. This translocation is found in 20% of DLBCL. In the following years over 15 proteins have been added to this family, each containing one or more BCL-2 Homology (BH) domain. Bcl-2-family proteins play central roles in cell death regulation and are capable of regulating diverse cell death mechanisms that encompass apoptosis, necrosis and autophagy [7,8].

In addition to chromosomal translocations as a mechanism for activation of the BCL-2 gene in human malignancies, changes to BCL-2 gene structure or copy number and many additional mechanisms contribute to elevated gene expression, which is estimated to occur in perhaps as many as half of all human cancers. Among the contributing mechanisms are (a) loss of endogenous microRNAs (miRs) that normally repress BCL-2 gene expression which has been documented in chronic lymphocytic leukemia, where the genes encoding miR15 and miR16 become deleted or inactivated by mutations in >70% of these leukemia, and gene hypomethylation, implying altered epigenetic regulation of BCL-2 in some malignancies [9,10].

Non-Hodgkin's lymphoma is the seventh most common cancer in clinic [11]. lymphoma can be treated with anti-apoptotic small molecule inhibitors of bcl-2 such as ABT-199 or venetoclax. However, small molecules generally present numerous side effects moreover; cancer becomes resistance to them after a month or two. Therefore, they offer no long-term solution or survival. Interfering with mRNA activities using RNAi could provide another alternative therapy as well [12-15]. It is well documented that the BCL-2 inhibitor drugs such as PNT2258 have beneficial effects in solid and non-Hodgkin's lymphoma tumours. The PNT2258 is a single-stranded DNA monocyclic phosphodiesterase contain a liposomal coating (SMARTICLE) which has therapeutically purpose via inhibition of BCL-2 [16]. The PNT2258 has anti-tumour activity in the xenograft models and has been safe and tolerant in Phase I clinical studies and has been reported as a highly effective drug in a pilot phase II clinical trials in patients with resist and recurrent non-Hodgkin's lymphoma [17-22].

However, there are limitations using this BCL-2 inhibitor drug, PNT2258. First, the high cost of liposomal carriers makes it less affordable; secondly, the drug delivery may not be efficient due to leaking and/or inefficient release of oligonucleotides in cytoplasm. Third, the liposomal components may also present some minor side effects such as thrombocytopenia (low count of platelets) and lymphocytopenia (low count of lymphocytes) as well as fatigue as reported in pilot phase II trial [17-22].

In this study, the liposomal coating of PNT100 has been dismissed and the DNA-based BCL-2 inhibitor has been delivered using novel Epigenic modification (confidential information). Therefore, Bicelin is being delivered directly without other compounds or chemicals as delivery system. Here, we first determine the in vitro cytotoxicity of Bicelin and then, evaluating its in vivo cytopenia, nephrotoxicity and hepatotoxicity effects of this drug in healthy animal model (Rats).

METHODS

Bicelin drug

PNT100 is a single-stranded DNA, 24-base oligonucleotide that has been designed by our colleague, Dr. Reza Sheikhnejad using

DNAi technology with over 20 international patents. This 24-base nucleotide was chemically synthesized on a solid phase column, cleaved and purified using HPLC. It was then freeze-dried before use. This oligonucleotide is designed specifically to regulate the activity of BCL2 but has no effect on other members of BCL2 family such as Bcl-xl, MCL-1 or Bcl-w [11]. The 24-base oligo is designed to hybridizes within untranscribed regulatory region of BCL2 thus interferes with oncogene transcription.

In vitro anti-cancer activity assay

Cancer cells, DLBCL (Diffuse Large B-Cell Lymphoma) were purchased from ATCC (LGC Standards, GmbH, Germany). The cells were seeded at 5,000 cells/well/200 μ l in a 96-well plate and the culture was maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 1% L-glutamine, 100 units/mL penicillin, and 100 μ g/mL streptomycin overnight. The media was replaced with fresh media containing plant extracts and incubated for 48 h in a humidified atmosphere of 95% air and 5% CO₂ at 37°C until the control cultures were confluent. The media was then removed and the plate was washed two times with Phosphate-Buffered Saline (PBS). Serum-free media (100 μ l) containing 0.5 mg/mL MTT dye was added into each well and incubated at 37°C for 2 h. The media with dye was removed, washed with PBS and the reactive dye was solved by addition of 100 μ l Dimethyl Sulfoxide (DMSO). The absorbance was read using an automatic plate-reader. All experiments were performed in triplicates.

Animal experiment

Ten, 3 months old male Wistar rats (279 \pm 10 g) were purchased from the animal house of Isfahan University of Medical Sciences. The animals were housed at the room temperature of 23°C-25°C. The rats had free access to water and chow. All procedures were approved by the Ethical Committee of Isfahan University of Medical Sciences. The rats were randomly divided into two groups named control and experimental group. The rats in experimental group were received Bicelin (20 mg/kg/day) for 5 days a week. The treatment was continued for 3 consecutive weeks. The control group was treated with saline only. At the end of 3 weeks, the animals were placed in metabolic cages for 4 hours to collect their urine.

Finally, the blood samples were collected and the rats were then sacrificed following the University approved ethical procedure. Briefly, the animals were anesthetized with chloral hydrate (450 mg/kg, intraperitoneally) (Merck, Germany). The, kidneys and liver tissues were fixed in formalin 10% to perform histological investigation using H&E staining. The kidney and liver tissues damage score (KTDS, LTDS) were recorded by two pathologist who were blinded to the study protocol. The score was assigned from 1 to 4 based on intensity of tissue damage while zero was considered as normal.

The blood samples were subjected to Cell Blood Count (CBC). The serum levels of Blood Urea Nitrogen (BUN) and Creatinine (Cr), Sodium (Na), Potassium (K), Aspartate aminotransferase (AST) Alanine Aminotransferase (ALT) and Anaplastic Lymphoma Kinase (ALK) also were determined. The Cr clearance (ClCr) was determined using clearance formula as; $ClCr = UF \cdot UCr / PCr$ where UF, UCr and PCr were assigned as urine flow rate, urine Cr concentration and serum level of Cr.

Statistical analysis

Data were reported as mean \pm SEM. The t-student test was applied for comparison between the groups. The KTDS between the groups were compared by non-parametric tests of Mann-Whitney U. The statistical P value was significant when it was less than 0.05.

RESULTS

The in vitro result (Figure 1) shows greater (≥ 8 fold) anti-cancer activity for Bicelin compare to liposomal PNT100 (PNT2258). The IC50 is calculated to be less than 10 $\mu\text{g}/\text{ml}$ ($\sim 1 \mu\text{M}$). Figure 2 shows that Bicelin is highly specific; it has little effect on lung and pancreatic cancer cells (10%-15%) and a modest in vitro activity on colon cancer (45%). But bcl2 inhibition seems to be most effective against diffuse large cell lymphoma which is known to have bcl2 translocation (Figure 3). Bicelin had almost no effect on normal cells (fibroblast cell line).

The blood test results demonstrated in Table 1 indicate that most

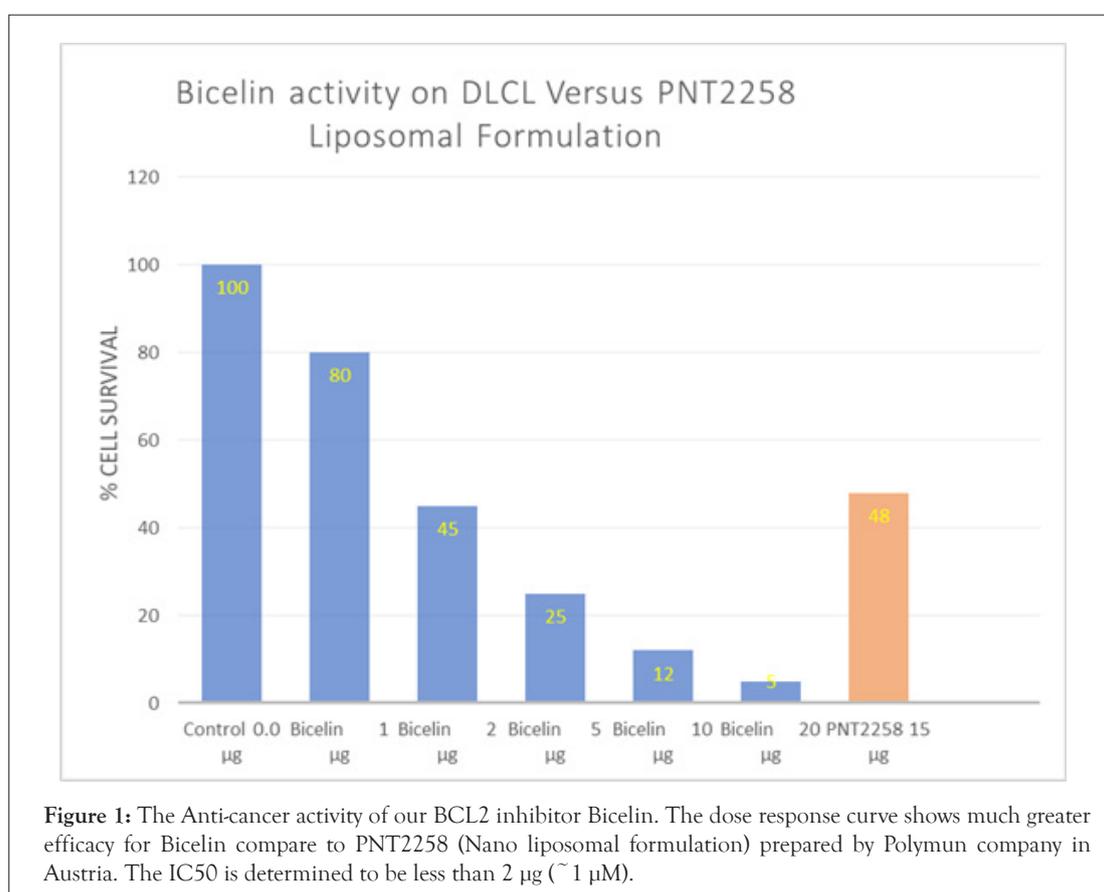
blood parameters including hemoglobin level were not significantly different when Bicelin treated rats were compared to the control group receiving only saline. We also observed no weight loss in rats receiving high dose of Bicelin after 15 consecutive injections (Figure 1). The Kidney Weight (KW), serum levels of Blood Urea Nitrogen (BUN), Creatinine (Cr), Aspartate aminotransferase (AST) Alanine Aminotransferase (ALT) and Anaplastic Lymphoma Kinase (ALK), and Urine Flow (UF), Cr Clearance (ClCr), and Sodium (ENa) and Potassium (EK) excretion fraction in two groups of animal treated with vehicle (control) and Bicelin. No significant differences were observed between the two groups measuring these parameters (Figure 4).

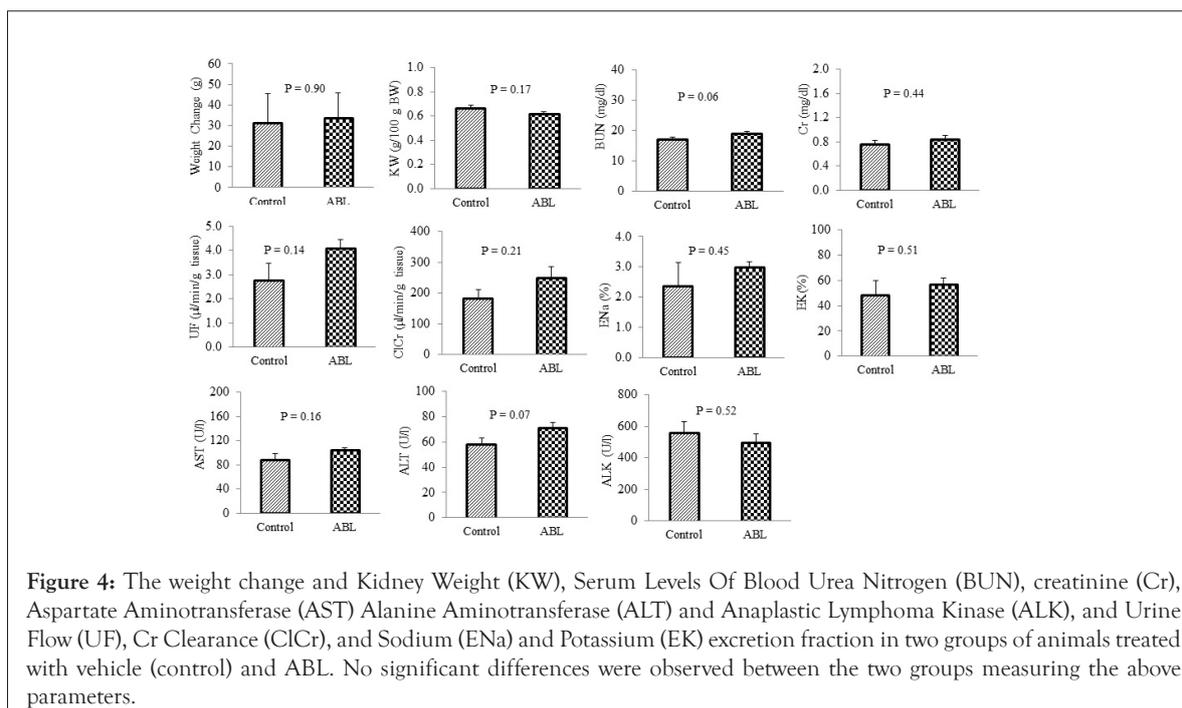
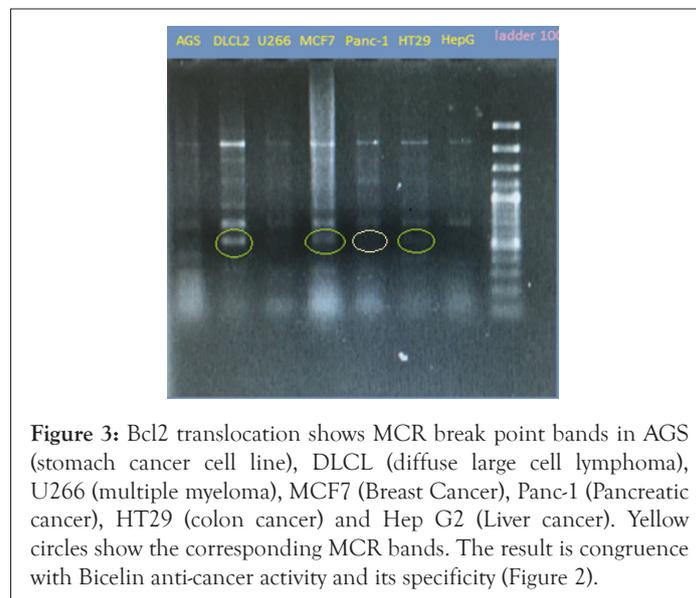
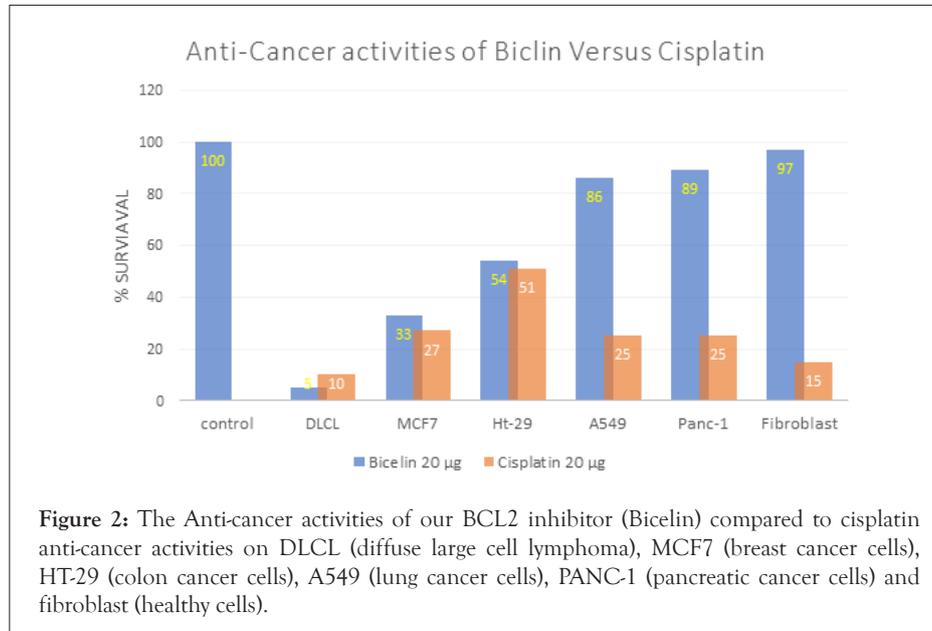
The pathological examination further proves the safety of our test compound, Bicelin. Figures 5 and 6 shows no kidney and liver tissue damages induced by Bicelin administration. These findings revealed that Bicelin is highly safe and has no toxicity effects on kidney and liver organs.

Table 1: The Cell blood count data in two groups of experiment. No significant differences were observed between the two groups, measuring the parameters are listed below.

Group	Hgb (g/dl)	Hct (%)	RBC	WBC/1000	Lymph	MCV	MCH	PLT 100000	MPV	BiT	BiD
SJC 28	12.74 \pm	38.78 \pm	7.024 \pm	8.62 \pm	81.42 \pm	55.46 \pm	18.18 \pm	7.34 \pm	6.96 \pm	0.71 \pm	0.066 \pm
SJC 28	SJC 28	SJC 28	SJC 28	SJC 28	SJC 28	SJC 28	SJC 28	1.68	0.081	0.08	0.017
ABL24	13.12 \pm	39.56 \pm	7.216 \pm	9.44 \pm	77.34 \pm	54.9 \pm	18.2 \pm	7.54 \pm	6.96 \pm	0.84 \pm	0.078 \pm
	0.31	0.98	0.225	0.86	1.53	0.65	0.27	0.316	0.09	0.03	0.007
P-value	0.51	0.61	0.63	0.75	0.38	0.78	0.96	0.91	1	0.19	0.55

Abbreviations: Hgb: Hemoglobin; Hct: Haematocrit; RBC: Red Blood Cells; WBC: White Blood Cells; MCV: Mean Corpuscular Volume; MCH: Mean Cell Hemoglobin; PLT: Platelets; MPV: Mean Platelet Volume; BiT: Behavioral inattention Test





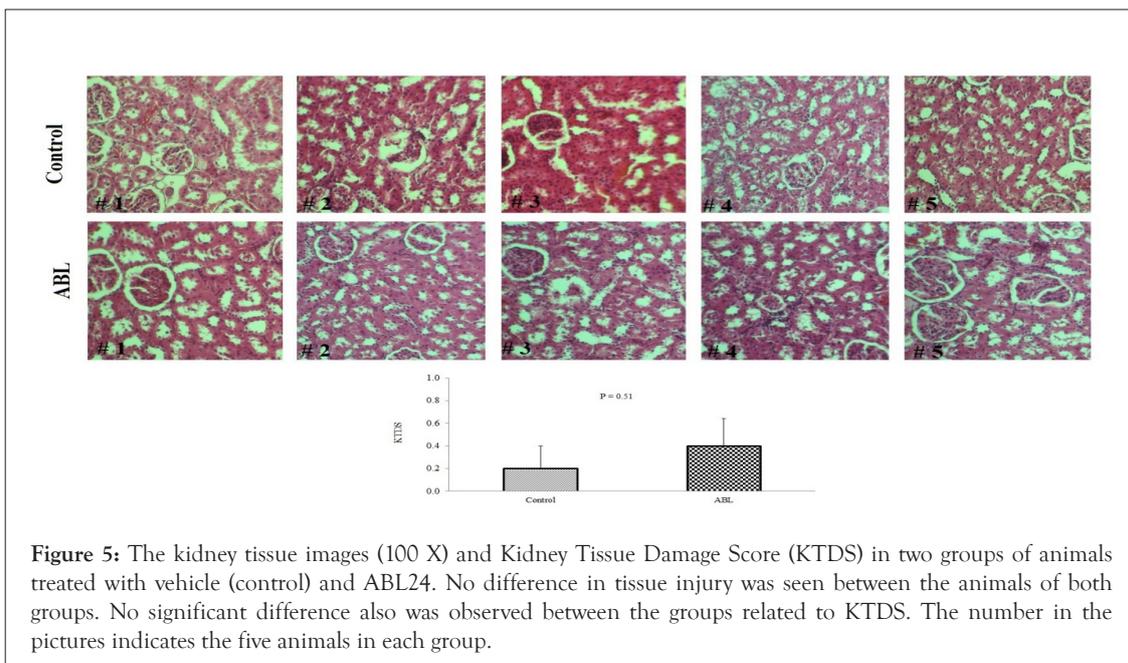


Figure 5: The kidney tissue images (100 X) and Kidney Tissue Damage Score (KTDS) in two groups of animals treated with vehicle (control) and ABL24. No difference in tissue injury was seen between the animals of both groups. No significant difference also was observed between the groups related to KTDS. The number in the pictures indicates the five animals in each group.

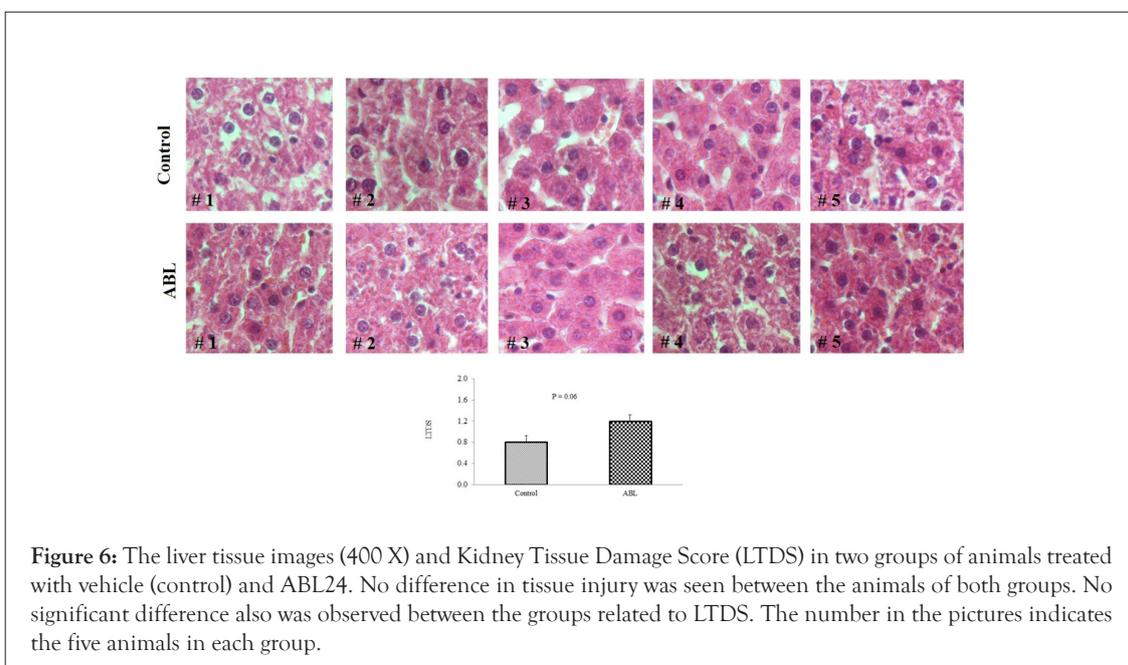


Figure 6: The liver tissue images (400 X) and Kidney Tissue Damage Score (LTDS) in two groups of animals treated with vehicle (control) and ABL24. No difference in tissue injury was seen between the animals of both groups. No significant difference also was observed between the groups related to LTDS. The number in the pictures indicates the five animals in each group.

RESULTS AND DISCUSSION

The results clearly demonstrate that bcl2 targeted Bicelin presents no toxicity when tested in vitro using several cancer cells as well as healthy fibroblast cells. Knowing that cell lines have zero tolerability for any toxic materials. Furthermore, the in vitro results also show that Bicelin is highly specific because it has little effect on lung and pancreatic cancer cells (10%-15%) and a modest in vitro activity on colon cancer (45%). Although bcl2 chromosomal translocation may happen in 10% to 20% of most cancers, but bcl2 inhibition seems to be most effective in non-Hodgkin lymphoma, Diffuse Large Cell Lymphoma (DLCL). The in vivo safety evaluation further indicated that Bicelin is highly safe and we expect no or very little side effect if tested in clinic. Table 1 as well as Figures 4-6 data profoundly proves our safety speculation. The in vivo results become more significant, considering that the rats were injected 15 consecutive times with no rest at a dose almost 20 times that of used in clinical trial. The

greatest advantage of using Bicelin rather than standard drugs used according to R-CHOP protocol would be in dosing schedule. There may be no need to interrupt the treatment after each cycle because of low blood cell counts (neutropenia or leukopenia) as well as low platelet count (thrombocytopenia) and low red blood count (anemia) that happen because of chemotherapy's effect on blood cells made in the bone marrow. Blood cell counts often reach their lowest level about 7 to 14 days after chemotherapy. Low blood cell counts are the most common and most serious side effect of chemotherapy. When it happens, the dose of chemotherapy is adjusted and the treatment is delayed until cytopenia improved. Increases the risk for infection. The Bicelin in vivo safety results show absolutely no change in blood cell counts. Therefore, we anticipate no interruption in patient treatment and the dose 120 mg/m² as determined in phase I clinical trial of PNT2258, can be escalated if needed. In conclusion, based on our in vitro and in vivo safety studies, our bcl2 inhibitor, Bicelin is much safer and

about 10-fold more effective than its liposomal form (PNT2258). Considering preclinical, phase I and II studies of PNT2258, Bicelin is expected to be very safe and effective in clinic.

CONCLUSION

Bicelin, a modified DNA oligonucleotide (24 bp), with strong anticancer activity is proven to be extremely safe. After 15 consecutive iv injections, no cytopenia, hepatotoxicity or nephron toxicity was observed in rats. We expect, promising efficacy with little or no side effects when tested in clinic.

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Availability of data and materials

The data and materials of in vitro study is available at Oshida Novel Pharmatech and the in vivo data is available at Department of Clinical Pathology, Isfahan University of Medical Sciences, Isfahan, Iran.

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Authors' contributions

First author, Reza Sheikhnejad (RS) has developed and formulated the experimental drug for this study, and carried out the in vitro tests as well. The second author, Farzaneh Ashrafi (FA) has directed the animal study and helped to design the protocol. The co-author, MN has designed and supervised the animal study; AT, has performed the pathology; BM and FM have carried out the animal study.

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