A B S T R A C T

Recent researches have demonstrated improved survival in oncologic patients treated with low molecular weight heparins (LMWHs) which are anticoagulant drugs. We evaluated “second generation” LMWH bemiparin and its in vitro anti-tumor effects on HepG2 hepatocellular carcinoma and MIA PaCa-2 cancer cells. The aim of the study is to investigate anti-cancer mechanism of bemiparin in HepG2 and Mia-Paca-2 cancer cells. Cytotoxic effects of bemiparin were determined by XTT assay. IC_{50} dose of bemiparin was found to be 200 IU/mL in the 48th hour in the MiaPaCa-2 cell line and 50 IU/mL in the 48th hour in the HepG2 cell line. CCND1 (cyclin D1), CDK4, CDK6, p21, p16, p53, caspase-3, caspase-9, caspase-8, Bcl-2, BID, DR4, DR5, FADD, TRADD, Bax, gene mRNA expressions were evaluated by Real-time PCR. Real-time PCR analysis showed that CCND1 expression was reduced in HepG2 dose the group cells when compared with the control group cells and p53, caspase-3, caspase p21, caspase-8 and expressions were increased in the dose group cells when compared with the control group cells. CCND1, CDK4 and CDK6 expressions were reduced in Mia PaCa-2 dose group cells when compared with the control group cells and p53 expression was increased in the dose group cells when compared with the control group cells. Other expressions of genes were found statistically insignificant both of cell lines. It was found that bemiparin in HepG2 and Mia PaCa-2 cells suppressed invasion, migration, and colony formation by using matrigel invasion chamber, and colony formation assay, respectively. In conclusion, it is thought that bemiparin indicates anti-tumor activity by affecting cell cycle arrest, apoptosis, invasion, migration, and colony formation on cancer cells.

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Research paper

Anti-tumor effects of bemiparin in HepG2 and MIA PaCa-2 cells

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1. Introduction

Bemiparin, which has mean molecular weight (3600 Da) and the longest half-life (5.3 h), called second-generation low-molecular-weight heparin (LMWH). Bemiparin is a member of LMWH which is new class of anticoagulants derived from commercial grade unfractioned heparin (UFH) (Martínez-González and Rodríguez, 2010; Planès, 2003). Although bemiparin demonstrates its anticoagulant effects via its anti-Xa activity, also other factors such as the release of tissue factor pathway inhibitors (TFPI) from endotelial cells can regulate this activity (Planès, 2003; Weitz, 1997; Hirsh et al., 2001). At the same time, heparin binds to tumoral cell proteins and macrophages with low affinity than other LMWHs or UFH (Morita et al., 2001; Gebska et al., 2002). This situation may be associated with decrease in anticoagulant potency and presumably dose–response (Planès, 2003).

Association between cancer and thrombosis is well defined for long years. It is reported that there is a two-way association in that presence of the tumor stimulates a hypercoagulable state in the host leading patients to thrombosis or in contrast to this situation, the induction of coagulation promotes the tumor progression. Focusing on these relation may have significant effect on survival of cancer patients (Falanga et al., 2003; Vignoli et al., 2011). It is also reported that venous thromboembolism (VTE) is responsible approximately 3.5% death of cancer patients (Khorana et al., 2007). LMWHs are frequently used for thromboprophylaxis in cancer patients with high risk for both venous and arterial thrombosis (Khorana et al., 2009). The gold standard treatment for acute DVT patient is low-molecular weight heparins (LMWHs) and vitamin K antagonists (VKAs) together with compression stockings. The most important benefit of this treatment reduces mortality by preventing pulmonary embolism (PE) (Polat et al., 2015).

One of the second generation LMWH, bemiparin has been recently utilized in clinical practice of either thromboprophylaxis or adjuvant therapy in oncological patients (Kakkar et al., 2010; Lecumberri et al., 2009).
The number of cells in matrigel matrix basement membrane formed by Step One Plus Real Time RT-PCR (Applied Biosystems, USA) protocol. RT-PCR assay was performed using gene-specific primer sequences of the genes used in this study.

### Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BAX</strong></td>
<td>F: AGAGGATGGTAAGCCGCCCT</td>
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<tr>
<td><strong>CASPASE-3</strong></td>
<td>F: GCAGCAACTCCTAAGAGAAAAC</td>
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<tr>
<td><strong>BCL-2</strong></td>
<td>F: TTGGCCCGTGTGGTTT</td>
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<tr>
<td><strong>GAPDH</strong></td>
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<tr>
<td><strong>CASPASE-9</strong></td>
<td>F: GCCTCTTACGCGCAAGATAGGA</td>
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<td><strong>P21</strong></td>
<td>F: TTGGACTCTCAGGGTTGCAAA</td>
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<tr>
<td><strong>PS3</strong></td>
<td>F: ATCTAAGCATGACACACCAT</td>
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<tr>
<td><strong>BID</strong></td>
<td>F: CCTACCCATTGACATGGAGAGA</td>
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<tr>
<td><strong>DR4</strong> (TNFRSF10A)</td>
<td>F: CCGGGAGAAGGAAACAC</td>
</tr>
<tr>
<td><strong>DR5</strong> (TNFRSF10B)</td>
<td>F: ACCATGCCCCGACATTCTTGT</td>
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<td><strong>FADD</strong></td>
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<td><strong>TRADD</strong></td>
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<td><strong>CDK4</strong></td>
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<td><strong>CDK6</strong></td>
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<td><strong>P16</strong></td>
<td>F: CAGTTAAGCTACGCCGATAGA</td>
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<tr>
<td><strong>CASPASE-8</strong></td>
<td>F: TCTGAGACATCTGTAGTCTG</td>
</tr>
</tbody>
</table>

(housekeeping gene) expressions to calculate relative expression ratios. Primer sequences used in this study were given in Table 1.

### 2. Material and methods

#### 2.1. Cell Culture

MIA PaCa-2 human pancreatic cancer cells and HepG2 hepatocellular carcinoma cells were seeded in DMEM medium supplemented with 2 mM-glutamine, penicillin (20 units/mL), streptomycin (20 μg/mL), and 10% (vol/vol) heat-inactivated fetal calf serum at 37 °C in a saturated humidity atmosphere containing 5% CO₂. MIA PaCa-2 and HepG2 cells were treated with 0.1 IU/mL, 0.5 IU/mL, 1 IU/mL, 10 IU/mL, 25 IU/mL, 50 IU/mL, 100 IU/mL, 200 IU/mL, 300 IU/mL bemiparin-dissolved in medium up to 72 h, in a time and dose dependent manner.

#### 2.2. Cell viability assay

Effects of bemiparin on cell viability and detecting of IC₅₀ dose in MIA PaCa-2 and HepG2 cells were performed by using trypan blue dye exclusion test and XTT [2,3-bis-(2-Methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] assay as indicated in manufacturers’ instruction. MIA PaCa-2 and HepG2 cells were seeded in 96-well plates at a number of 3 x 10⁴ cells/well and incubated for 24 h without reagent. The cells were treated with different concentrations of bemiparin and incubated for 24, 48, and 72 h. Then XTT mixture was added as recommended by supplier assessed cell viability. Formazan formation was quantified spectrophotometrically at 450 nM (reference wavelength 630 nM) using a microplate reader. Viability was calculated using the background-corrected absorbance as follows:

Cell Viability(%) = A of experiment well/A of control well x 100

#### 2.3. RNA isolation and Real Time-PCR

Total RNA was isolated from the cells exposed to IC₅₀ (inhibitory concentration where 50% of the cells die) doses of bemiparin using Trizol Reagent (Invitrogen, USA) according to manufacturer instructions. cDNA synthesis from RNA template was performed via reverse transcription by using Transcriptor First-Strand cDNA Synthesis Kit (Roche, Germany) according to manufacturer protocol. p21, p16, p53, caspase-3, caspase-9, caspase-8, FADD, TRADD, Bax, CCND1 (cyclin D1), CDK4, CDK6, Bcl-2, Bid, Dr4, Dr5 gene expression analysis was performed by Step One Plus Real Time RT-PCR (Applied Biosystems, USA) according to the SYBR Green qPCR Master Mix (Thermo Scientific, USA) protocol. RT-PCR assay was performed using gene-specific primers. The expression results were regulated to the GAPDH gene (housekeeping gene) expressions to calculate relative expression ratios. Primer sequences used in this study were given in Table 1.

#### 2.4. Determination of cell invasion and migration effect of bemiparin

Effects of bemiparin on invasion activities of HepG2 and MIA PaCa-2 cells were determined via the Bio Coat Matrigel Invasion Chamber guide (BD Biosciences). HepG2 and MIA PaCa-2 cells with serum-free DMEM-medium were seeded on the upper surface of the chamber with Matrigal-coated filter inserts and serum-containing DMEM medium (500 μL) was added to the lower chambers. The control and dose group cells were then incubated at 37 °C for 24 h. After the incubation, filter inserts were removed from the wells. The cells exist on the upper surface of the filter were wiped with a cotton swab. Filters were fixed with methanol for 10 min and stained with crystal violet. The cells that invaded the filter were stained using a light microscope. Each experiment was performed in triplicate. Percentage of invasion was calculated as follows:

\[
\text{Invasion} (\%) = \frac{\text{The number of cells in matrigel coated basement membrane}}{\text{The number of cells in control membrane}} \times 100
\]

#### 2.5. Colony assay

To define colony formation effects of bemiparin in HepG2 and MIA PaCa-2, the cells were seeded in six-well plates at a density of 10⁴ cells per well. The medium was renewed every 3 days during three weeks until visible colonies formed. Colonies were fixed in methanol for 10 min and stained with crystal violet to make them visible. Thus, colony numbers of control and bemiparin treated cells were compared.
2.6. Statistical analysis

The gene expression analysis by determined Real Time PCR were made using the ΔΔCT method and quantitated with a software program. The comparison of the control and dose groups has been defined with “Volcano Plot” analysis, from “RT² Profiles™ PCR Array Data Analysis”, which is assessed statistically using the “Student t-test”. All the results were expressed as mean ± standard error and other statistical analysis of the study have been evaluated with the SPSS 17.0 statistical analysis program. For all of the analyses, P-value of less than 0.05 was considered statistically significant. (P < 0.05 is statistically significant).

3. Results

3.1. XTT assay

Upon treatment with bemiparin, viability of MIA PaCa-2 and HepG2 cells were assessed by XTT assay. Decrease in viability in MIA PaCa-2 and HepG2 cells were observed in a time- and dose-dependent manner. The expression changes of genes are evaluated by treating MIA PaCa-2 and HepG2 cells were observed in a time- and dose-dependent manner. Decrease in viability in MIA PaCa-2 and HepG2 cell line. (Fig. 1B).

3.2. Real-Time-PCR

The expression analysis of CCND1 (cyclin D1), CDK4, CDK6, p21, p16, p53, caspase-3, caspase-9, caspase-8, DR4, DR5, Bcl-2, BID, FADD, TRADD, Bax was determined by Real Time RT-PCR according to the SYBR Green qPCR Master Mix (Thermo Scientific) protocol. Real-Time PCR analysis demonstrated that CCND1 expression was reduced in HepG2 dose the group cells when compared with the control group cells and p53, caspase-3, caspase p21, caspase-8 and expressions were increased in the dose group cells when compared with the control group cells (Table 2; P < 0.05). Other expressions of genes were found statistically insignificant (P > 0.05). Other expressions of genes were found statistically insignificant (P > 0.05).

3.3. Migration and invasion assay

According to the results of Matrigel invasion chamber assay, the cell invasion was significantly inhibited in the bemiparin treatment groups, compared with the control groups (Fig. 2A, B). Invasion data of the four groups in HepG2 and MIA PaCa-2 cell lines were shown as follows, respectively: control groups (39 ± 3.6 and 44 ± 4.72)% and bemiparin doses groups (24 ± 2.4 and 21 ± 2.9)% (Fig. 2A, B).

3.4. Colony formation assay

Colony formation analysis was performed using colony formation assay. It was observed that colony formation decreased in the bemiparin-treated group, compared with the control groups in the HepG2 and MIA PaCa-2 cells (Fig. 3A, B). Colony formation data of the four groups in HepG2 and MIA PaCa-2 cell lines were shown as follows, respectively: control groups (32 ± 1.2 and 485.6 ± 9.45) % and bemiparin dose groups (24 ± 1 and 353.6 ± 10.69)% (Fig. 3A, B).

4. Discussion

According to recent studies related to LMWH suggest that LMWHs are effective for the conservation and treatment of venous thrombosis in oncologic patients and also have beneficial effects on survival of these patients (Vignoli et al., 2011; Kuderer et al., 2007; Lazo-Langner et al., 2007). Although there were clinical and preclinical studies in the literature about bemiparin and its anti-tumor and anti-metastatic effects, the underlying the possible effecting mechanisms of bemiparin are not known very well. An important relation may exist between progresion of tumors, invasion, angiogenesis, metastasis and cancer coagulopathy (Norrby, 2006; Boccaccio et al., 2005; Yu et al., 2005). The effects of heparins, especially “first generation” LMWH, were researched with in vitro and in vivo studies. Furthermore, recent studies associated with second generation LMWH were analyzed for their composition of polysaccharidic chains, anti-cancer activities and better efficacy/safety profiles (Khorana et al., 2003; Norrby, 2006; Marchetti et al., 2008).

Table 2

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change</th>
<th>Gene</th>
<th>Fold change</th>
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<tr>
<td>HepG2</td>
<td>MIA PaCa-2</td>
<td></td>
<td></td>
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<tr>
<td>1</td>
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<tr>
<td>2</td>
<td>P21</td>
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<td>5</td>
<td>CASPASE-8</td>
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In the current study, cytotoxic effects of Bemiparin in HepG2 and MIA PaCa-2 cells were determined in a time- and dose-dependent manner via XTT method. Each cells were treated with Bemiparin for 24, 48 and 72 h. Data showed that IC_{50} dose of bemiparin was calculated 200 IU/mL on 48th hour in the MIA PaCa-2 pancreatic cancer cell line and 50 IU/mL on 48th hour in the HepG2 hepatocellular cell line. After obtained IC_{50} doses of bemiparin for each cells, MIA PaCa-2 and HepG2 cells were treated these doses for gene expression analysis by real-time PCR, migration and invasion assay, colony formation assay.

CCND1 (cyclin D1) protein serve as a regulator protein in cell cycle’s G1-S checkpoint. It plays role in cell cycle arrest, activation of oncogenes or inhibition of tumor suppressor genes by interacting with special cyclin-dependent kinases (CDKs). Various cyclin/CDK complexes were determined in mammalian cells and they have functions in modulating distinct cell cycle transitions. Cyclin D-CDK4–6 which is the one of cyclin/CDK complex, involve in G1 cell cycle phase progression. In various tumor types, oncogenic role of CCND1 has been determined. Studies in many several cancer types suggested that overexpression or amplification of Cyclin D1 cause tumor growth (Peurala et al., 2013; Musgrove et al., 2011). Deregulation of CDK4 activity in various human tumors was observed and determined that CDK4 play crucial role for distinct oncogenic transformation processes. As a result of this, different cancer cells may have high CDK activity (Paternot et al., 2010). Real-time analysis in present study showed that CCND1 expression was significantly reduced in bemiparin-treated HepG2 cells when compared with the control group cells. For CDK4 and CDK6, we didn’t found significant relation in bemiparin-treated HepG2 cells. On the other hand, IC_{50} of bemiparin significantly reduced mRNA expressions of CCND1, CDK4 and CDK6 in MIA PaCa-2 cells according to control cells. Our results suggest that bemiparin have a potential effect on cell cycle control in HepG2 and MIA PaCa-2 cells by down regulating CCND1, CDK4 and CDK6. Effect of bemiparin on cell cycle is firstly shown by our study in both cell lines.

p53, the guardian of the genome, which is a transcription factor that play a crucial role in cell cycle control and programmed cell death (Struckhoff et al., 2010). p53 plays a pivotal role in genomic stability, apoptosis and inhibition of angiogenesis, and have function in anti-cancer mechanisms. Apoptosis which is a complex process, is regulated through extrinsic and intrinsic signaling pathway (Igney and Krammer, 2002). In apoptotic mechanism, p53 perform its role by transactivation abilities via binding apoptosis-related target genes and induced transcription of apoptosis related genes (Fridman and Lowe, 2003; Hassan et al., 2012). According to our findings, IC_{50} dose of bemiparin increased the mRNA expression of p53 gene in both cells significantly compared with the control cells. We also observed that mRNA expression of p21, downstream target of p53, were significantly increased in bemiparin-treated HepG2 cell line according to control group. In terms of apoptosis related genes, significant increased mRNA expression levels of caspase-3 and caspase-8 were obtained in HepG2 cells. But there was no significant association for apoptosis-related genes in bemiparin-treated MIA PaCa-2 cells. Our findings suggest that bemiparin may effect cell cycle progression and apoptotic pathway via increasing p53, p21, caspase-3 and -8 gene expressions in HepG2 hepatocellular carcinoma cells. On the other hand, bemiparin may suppress only cell cycle progression.
progression by increasing p53 mRNA expression and down-regulating CCND1, CDK4 and CDK6, in MIA PaCa-2 pancreatic cancer cells.

Cell migration, invasion are crucial steps in various physiological processes such as morphogenesis, angiogenesis, wound healing and inflammation (Bozzuto et al., 2010). Invasion and migration are critical events for tumor progression and tumor recurrence (Lavictoire et al., 2003). According to matrigel-chamber results, the invasion of hepatocellular and pancreatic cells were reduced in the bemiparin treated group, compared to the normal group. Invasion data of the four groups in HepG2 and MIA PaCa-2 cell lines were shown as follows, respectively: control groups (39 ± 3.6 and 44 ± 4.72)% and bemiparin dose groups (24 ± 2.4 and 21 ± 2.9)%. These results suggested that invasion and migration of cancer cells were inhibited by bemiparin treating. Colony formation data of the four groups in HepG2 and MIA PaCa-2 cell lines were shown as follows, respectively: control groups (32 ± 1.2 and 485.6 ± 9.45)% and bemiparin dose groups (24 ± 1 and 353.6 ± 10.69)%. Colony formation test results demonstrate that colony numbers in both of dose groups were suppressed. These results suggest that bemiparin reduced cell invasion, migration and colony formation capacity in HepG2 hepatocellular cells and MIA PaCa-2 pancreatic cancer cells.

Vignoli et al. showed that in vitro characterization of the antiangiogenic, antimigratory and capillary-like tube formation capacity of the second generation LMWH bemiparin in an in vitro model. They showed effects of bemiparin on Human microvascular endothelial cell line-1 (HMEC-1), Human breast cancer cell line MDA-MB-231, Human small cell lung cancer (SCLC) cell line H69, Human promyelocytic leukemia cell line NB4. They also evaluated effects of bemiparin on angiogenesis the proangiogenic factors VEGF and FGF-2 in this study. They have showed that both second generation LMWH and new ULMWH including bemiparin display an antiangiogenic activity against tumor derived products. These findings demonstrated possible activating mechanisms of bemiparin in cancer cells (Vignoli et al., 2011). Guler et al. investigated effects of several LMWHs on viability of human umbilical vein endothelial cells and reported that bemiparin was associated with a more pronounced effect on reducing viability than nadroparin and enoxaparin (Guler et al., 2013). Our results are supporting these results associated with migration and also suggesting that bemiparin have anti-proliferative anti-invasive, anti-migratory roles and suppressing colony formation capacity in hepatocellular and pancreatic cancer cells. These are the first obtained results about bemiparin and its effects on hepatocellular and pancreatic cancer cells in vitro.

In conclusion, many in vitro and in vivo researches on novel agents and biomarker molecules that have been researched in cancer treatment, focus on specifically inducing apoptosis and cell cycle arrest in cancer cells and inhibiting invasion, angiogenesis, migration and metastasis. Therefore, novel biological and chemical agents can be developed and in hepatocellular and pancreatic cancer cell progression and treatment. Our results show that, bemiparin inhibits cell proliferation by inducing cell cycle arrest and apoptotic mechanisms and also decreases invasion, migration and colony formation in HepG2 hepatocellular carcinoma and MIA PaCa-2 pancreatic cancer cell line. We presented the effect mechanisms related to apoptosis, cell cycle arrest and invasion of bemiparin in these cancer cells. Bemiparin may be a novel agent for treatment of hepatocellular carcinoma and pancreatic cancer as a single agent or in combination with other drugs, components or molecules. Therefore, more detailed studies in vitro and in vivo animal models should be designed to determine a safe dose with the best effects of bemiparin.
References


