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APPENDIX F

Regulation of the Biological Activity of Fibroblast Growth Factor by Enoxaparin *in Vitro*

Investigation Report

Regulation of the Biological Activity of Fibroblast Growth Factor by Enoxaparin™ in Vitro

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Introduction

Acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF) are potent stimulators of both DNA synthesis and cellular division in a variety of normal diploid mammalian cells that are derived from mesoderm or neuroectoderm. These include endothelial cells, fibroblasts, smooth muscle cells, oligodendrocytes, astrocytes, and retinal epithelial cells. (7,14,23)

Formation of new capillaries by endothelial cells involves a variety of complex cellular activity, including destruction of the basement membrane, migration, division and the subsequent reconstitution of the capillary structures (7). FGF stimulates many of these processes both in cellular cultures and in vivo. Application of FGF to the rabbit cornea stimulates directed neoangiogenesis. These growth factors are naturally released by the mononuclear phagocytes following inflammation or hypoxia.(15).

Recent investigations have shown that the application of FGF to the hypoxic myocardium induces the growth of new functional capillaries in the experimental animal, (1,2,9,12,17,28) an observation which could be confirmed in patients with CHD (21). Crude extracts of heparin bind FGFs, which can be purified by heparin affinity chromatography (11). Both forms of FGF are protected from degradation and damage when associated with heparin or the heparin-like carbohydrates of heparin-sulfate proteoglycansi(9). Glycosaminoglycans bind to FGFs with a high degree of specificity and induce interaction with the FGF receptors (25,26,27) thereby regulating the growth factor activity (6,18, 19, 24, 25, 26, 27.)

In vitro heparin potentiates the biological activity of aFGF, increasing its potency to that of bFGF (7). No additional activation of bFGF can be achieved by heparin (10). It is, however, interesting, that when heparin was added to endothelial cells in the absence of FGF, proliferation was inhibited (3). Growth was also inhibited by heparin in the presence of FGF if the heparin concentration was increased (10).

Heparin consists of a complex mixture of polysaccharides varying in size between 5000-30000 daltons. Sudhalter et al. studied the effects of size, sulfation, and anticoagulant activity on the potentiation of acidic fibroblast growth factor by heparin, and it was also found that a minimum chain length and degree of sulfation are required for potentiation (3). However, this issue remains controversial Perhaps the safest conclusion at present is that neither the length of the heparin fragment nor the stoichiometry of FGF binding to heparin are the sole determinants of activity (6). This observation suggests a role for heparin fragments (apart from its anticoagulant activity) in regulating the growth of vascular endothelial cells by modulating the stability and activity of FGF in vivo.

It is`, therefore, of great interest to test growth promoting effect of heparin fractions on aFGF induced cell proliferation in vitro, because from these tests conclusions concerning the activity of the fractions for the treatment of coronary heart disease can be drawn.

Experimental design

1. Enoxaparin fractions

12 low molecular heparin fractions of different sizes and with different anticoagulant activity were tested.:

1. WSD 3093-Hexasaccharides	2. WSD 3093-Octasaccharides
3. WSD 3093-Decasaccharides	4. WSD 3093-Dodecasaccharides
5. WSD 3093-<Hexadecasaccharides	6. WSD 3093->Hexadecasacharides
7. DIA 2844-Hexasaccharides	8. DIA 2844-Octasaccharides
9. DIA 2844-Decasaccharides	10. DIA 2844-Dodecasaccharides
11. DIA 2844-<Hexadecasaccharides	12. DIA 2844->Hexadecasacharides

2. Materials and Methods

FGF

Recombinant human acidic fibroblast growth factors (aFGF Cat-No.PT 100-17A) was obtained from NatuTec, GmbH Frankfurt, FRG

BHK-21

The BHK-21 cell line (baby hamster kidney cells) was obtained from Cell Line Service, Heidelberg, FRG

- Growth medium

MEM (EAGLE) with Glutamax-I (Gibco, Cat.-No. 41090-028), to which 10% calf serum, supplement, and antibiotics have been added:

Penicillin / Streptomycin	100 IU/ml / 100 UG/ml
Sodium Bicarbonate	0.075%
Sodium Pyruvate	1 mM
MEM non-essential amino-acids	1x
HEPES	10 mM

- Starvation medium and stimulus medium

MEM (EAGLE) with Glutamax-I (Gibco, Cat.-No. 41090-028), to which supplement and antibiotics have been added:

Penicillin / Streptomycin	100 IU/ml / 100 UG/ml
Sodium Bicarbonate	0.075%
Sodium Pyruvate	1 mM
MEM non-essential amino-acids	1x
HEPES	10 mM

Procedure

. The cells are trypsinizated as for regular subculturing.

. The cell count is determined with a Neubauer chamber, and from the result the appropriate number of cells/ml mixed with the growth medium.

. The concentration of cells to be seeded is adjusted by thinning out the cell suspension to the total volume necessary for distribution throughout the 96-well microtiter plate.

. The cells are seeded into a 96-well microtiter plate with a flat under surface, using the previously determined concentration necessary to seed 200 µl in each well. The cell suspension must be continuously swirled around to ensure that, by thorough mixing, the suspension achieves an identical concentration in each well.

. The microtiter plate is placed in a CO_2 incubator at 37°C with a humid atmosphere and 5% CO_2.

. After a growth phase of 1 -2 days, the plate is removed from the incubator and the medium replaced by starvation medium:
-The medium is completely removed from the wells and discarded.
-200 µl fresh starvation medium is added to each well.
- Culture is continued in the incubator for a further 24 hours.

. After 24 hours the starvation medium is replaced by stimulus medium:
-The medium is completely removed from the wells and discarded.
-200 µl fresh stimulus medium is added to each well.
-20 µl of concentrated solution in various dilutions is added to the medium as a test substance.
-For the four-fold determination, the required test solutions at various concentrations are prepared by dilution with stimulus medium to which 1% FCS have been added. The number of dilution steps and the required volumes of the dilutions are obtained from the calibration plan of the microtiter plate. The schemes for pipetting and diluting (the concentration increases logarithmically) are added separately for each investigation.

- The microtiter plate with the test substances is replaced in the incubator for 24 hours.

- At the end of this time, the cells are marked over a period of 4 hours by addition of [methyl-^{3}H] thymidine (Amersham, 5 mCi/5 ml, TRK 300) with a final concentration of 1 µCi per well.
The plate is replaced in the incubator for a further 4 hours.

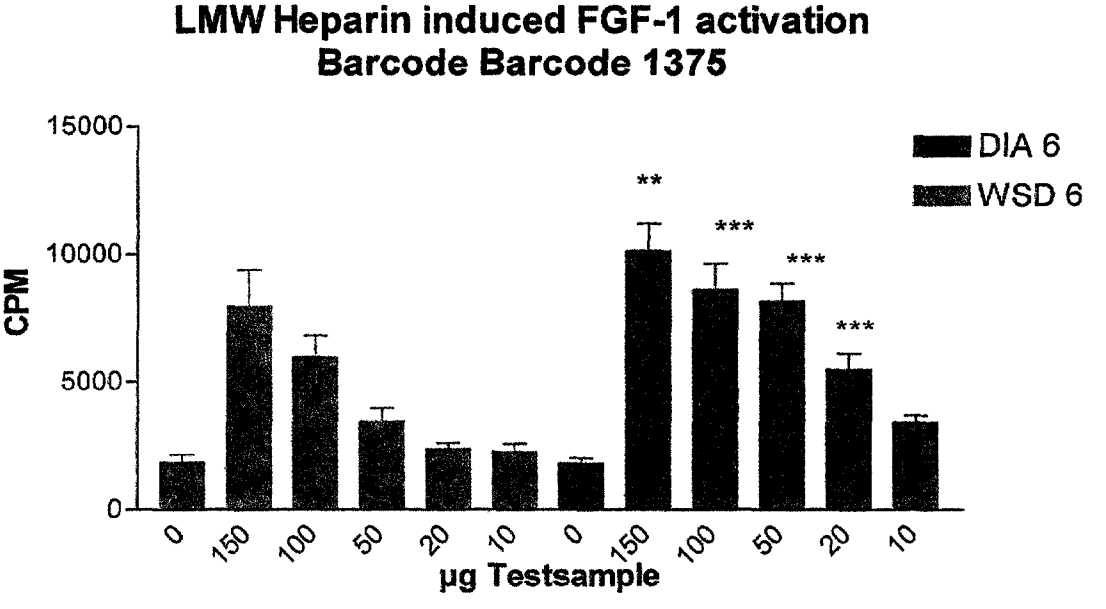
- The marked medium is completely removed from the wells in the plate with FilterMateTM Cell Harvester through a UniFilterTMPlate of 96 format with an integrated GF/C-Filter and discarded.

- 100µl 0.05% trypsine / 0.02% EDTA solution is pipetted into each well of the microtiter plate, and after 10 min. incubation at room temperature the cells of the 96

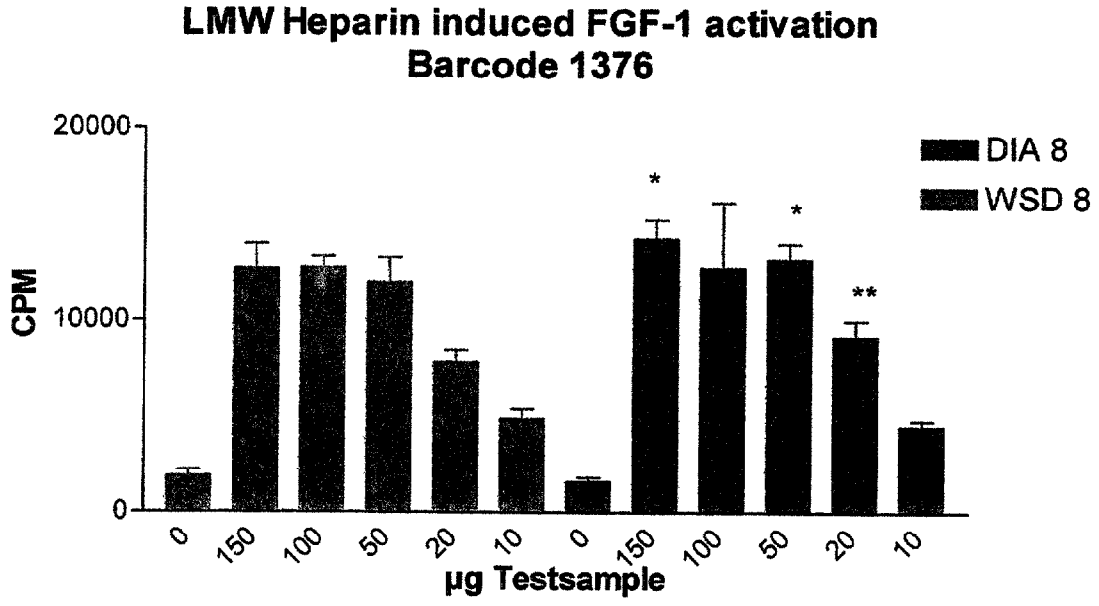
Results

1. Comparison of WSD and DIA compounds at increasing doses

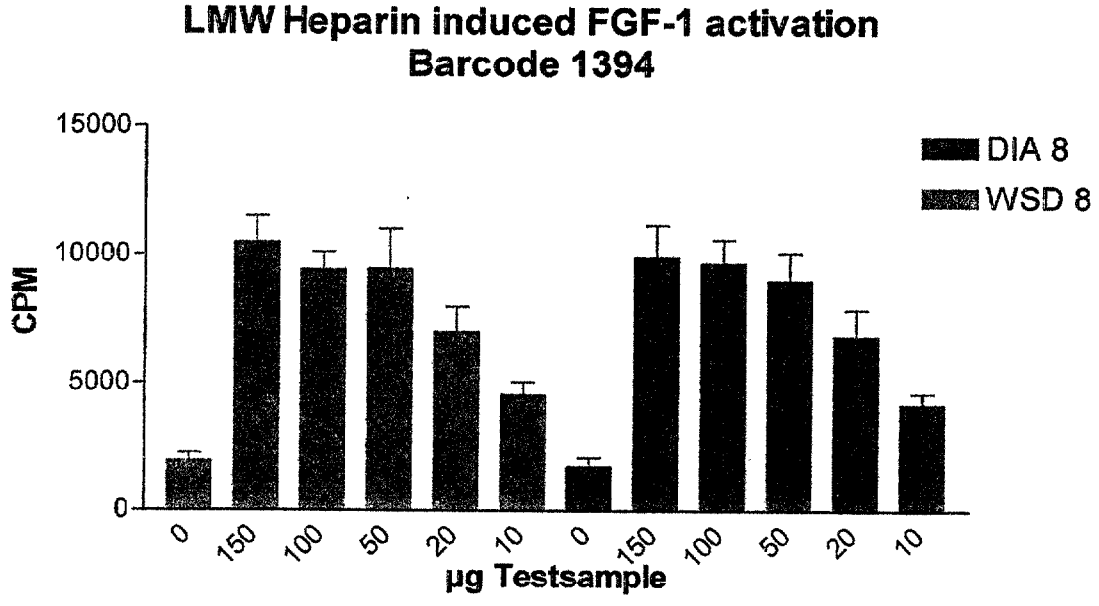
In the first series of experiments DIA and WSD compounds of the same chain length were compared in the range between 10 and 150 µg of the test substance at a constant dose of 1ng aFGF on single plates. Because of the relative high standard deviations obtained in cell culture tests each concentration was analysed in 8 replicates. Mean values and standard deviations were calculated. The values of DIA and WSD samples at equal doses were compared by statistical analysis (t-Test).

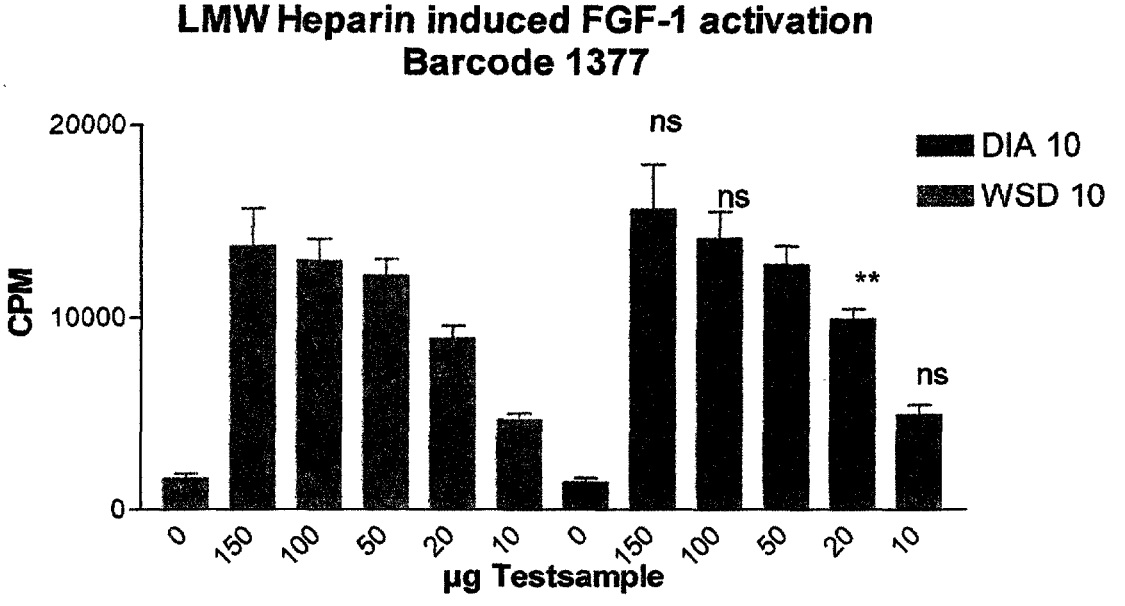


1a. **WSD-6 versus DIA-6.** The DIA compounds are significantly more active. However we obtained non significant differences in 4 other assays.

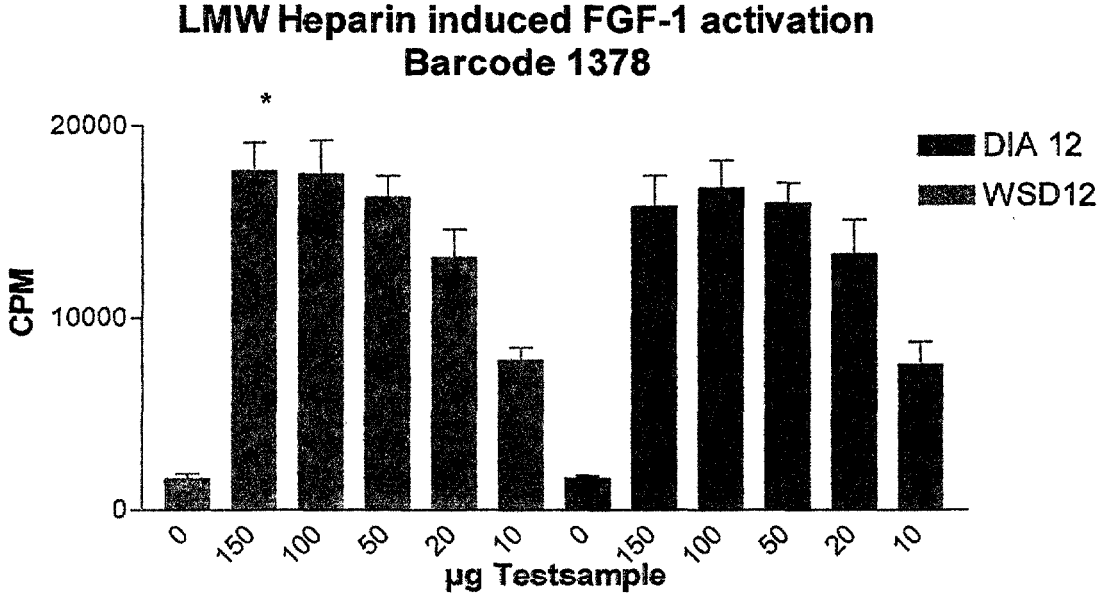


1b WSD-8 versus DIA-8. The DIA compounds are significantly more active. However the level of significance is lower compared to the differences measured with the hexasaccharides and could not be found in all assays performed.

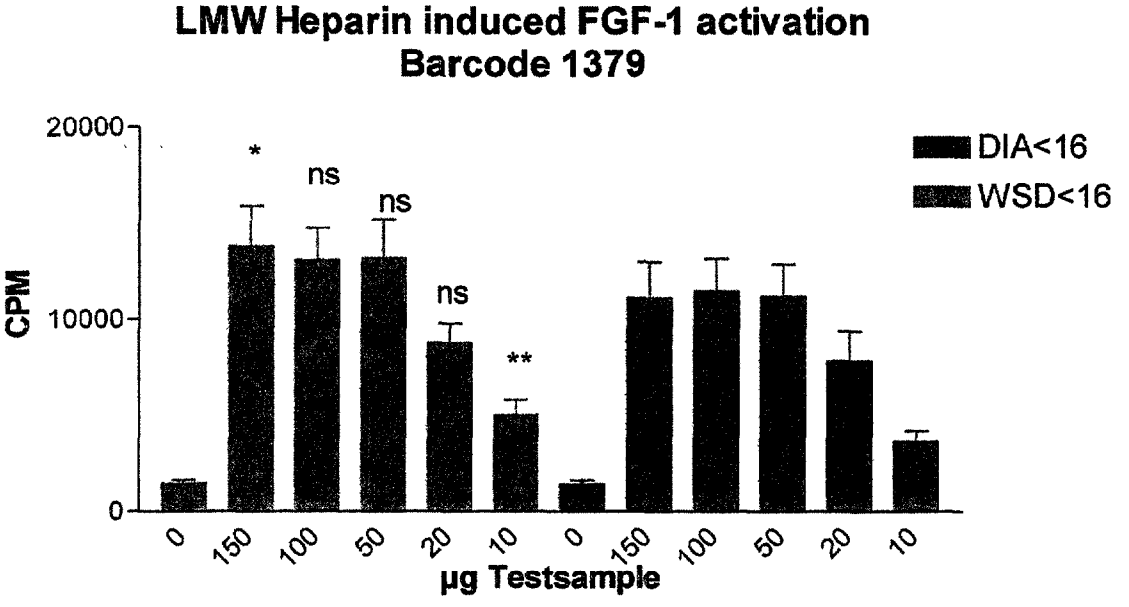




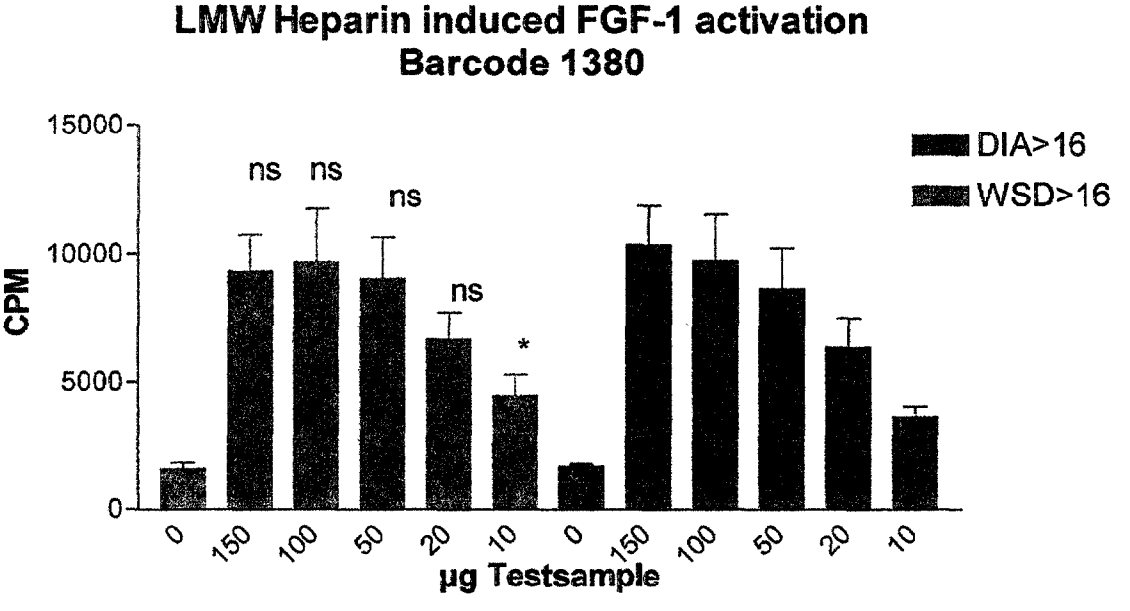
1c. DIA 10 versus WSD 10 The DIA compounds are significantly more active. However the level of significance is lower compared to the differences measured with the hexasaccharides and could not be found in all assays performed



1d. DIA12 versus WSD 12. With this assay no difference could be measured. Although at the 150 µg dose of the WSD compound induced a higher cell proliferation. However the fact that with the DIA samples a plateau is reached at lower concentrations compared to the WSD samples can be explained that the DIA samples are more active.



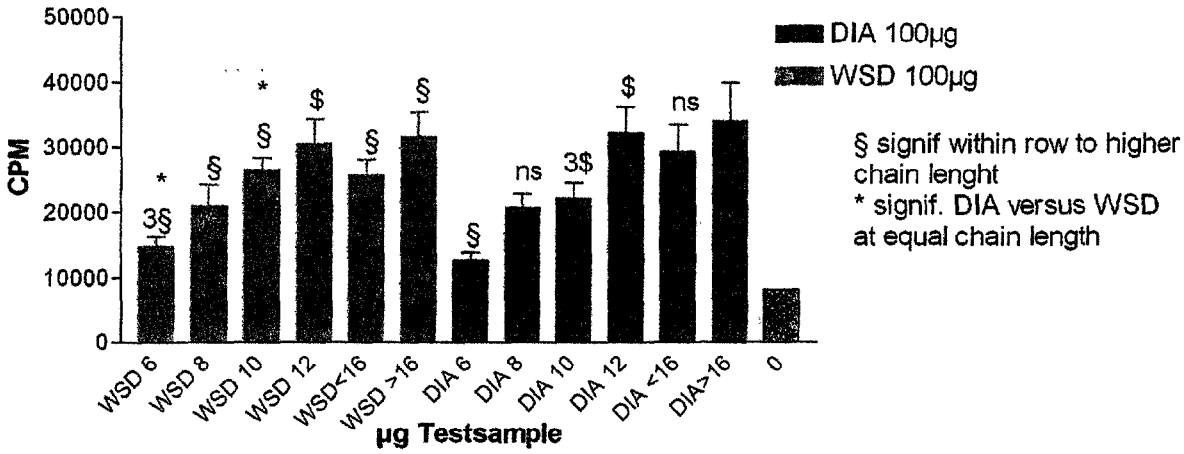
1e DIA <16 versus WSD<16



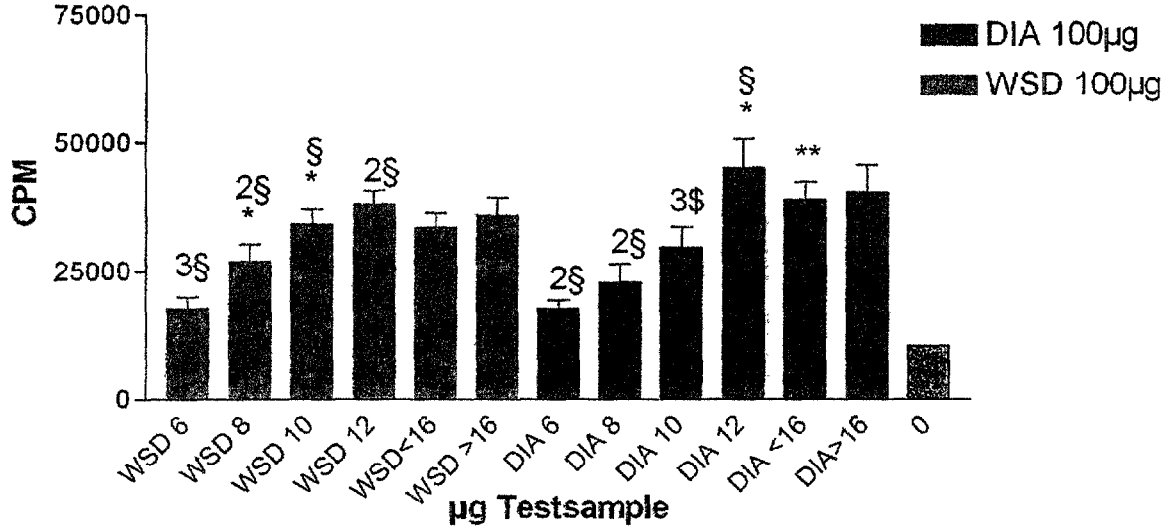
2. Comparison of all samples on one plate at a single dose

In order to get more information about the influence of the chain length on the activity we performed assays in which all samples were analysed at one concentration on one plate. Two different cell concentrations $4x10^4$, (barcodes 1405-1409) and $8x10^4$, (barcode 1410-1414) were tested. As shown in the graphs (barcodes 1404-1414) our previous results, showing a clear increase in activity towards higher chain length (6-12) of both DIA and WSD samples were confirmed. In addition we observed a decease of thymidine incorporation with the <Hexadecasaccharides in comparison to the dodecasaccharides, with both the DIA and the WSD compound.

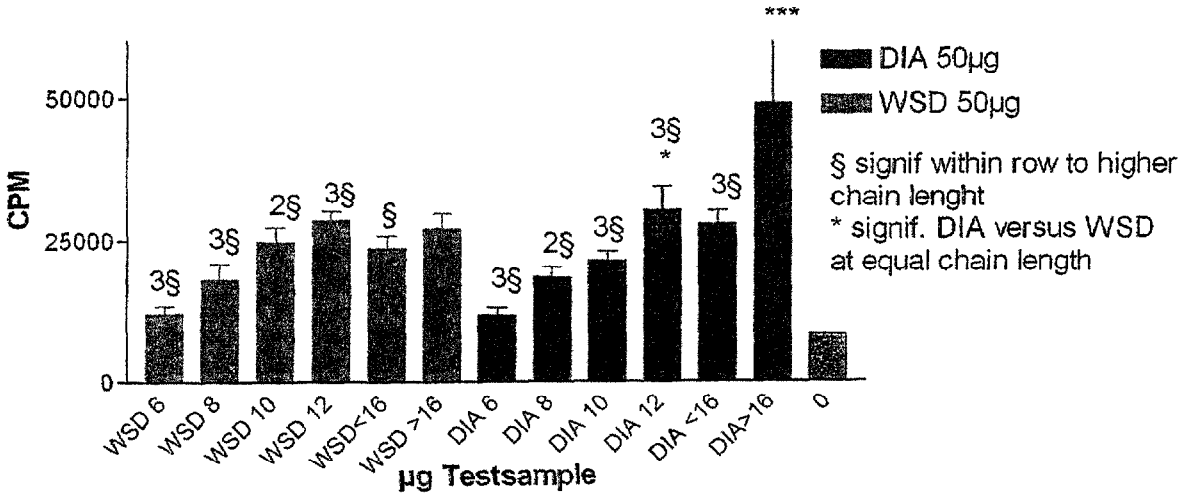
LMW Heparin induced FGF-1 activation
Barcode 1406



LMW Heparin induced FGF-1 activation
Barcode 1411

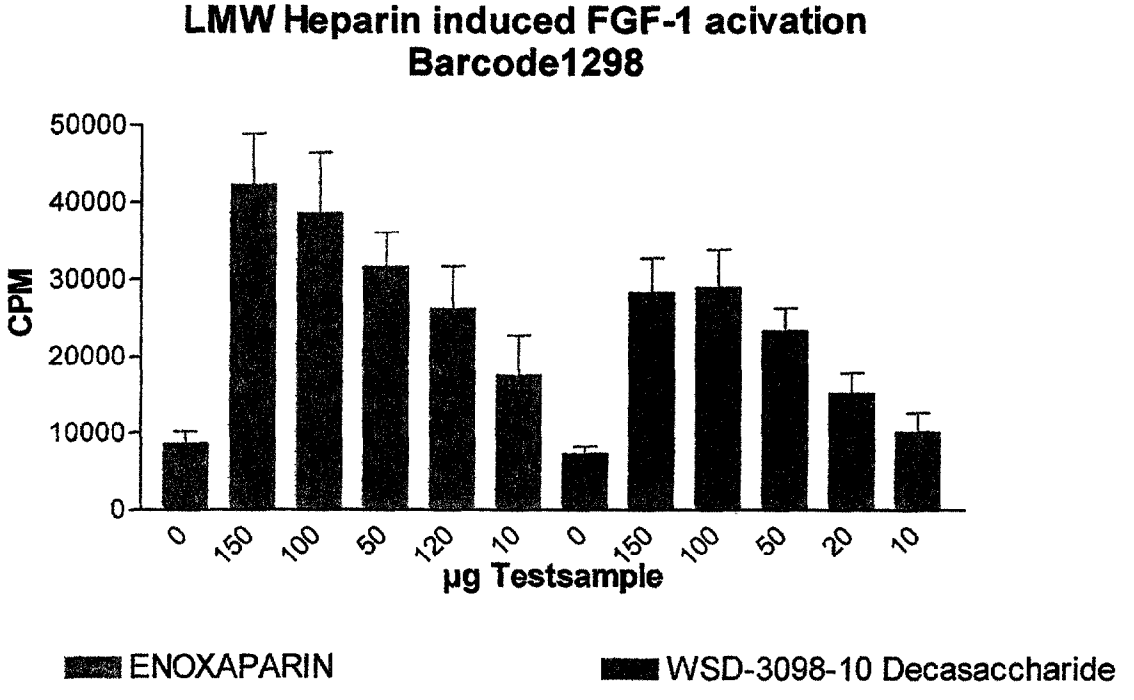


LMW Heparin induced FGF-1 activation
Barcode 1 407

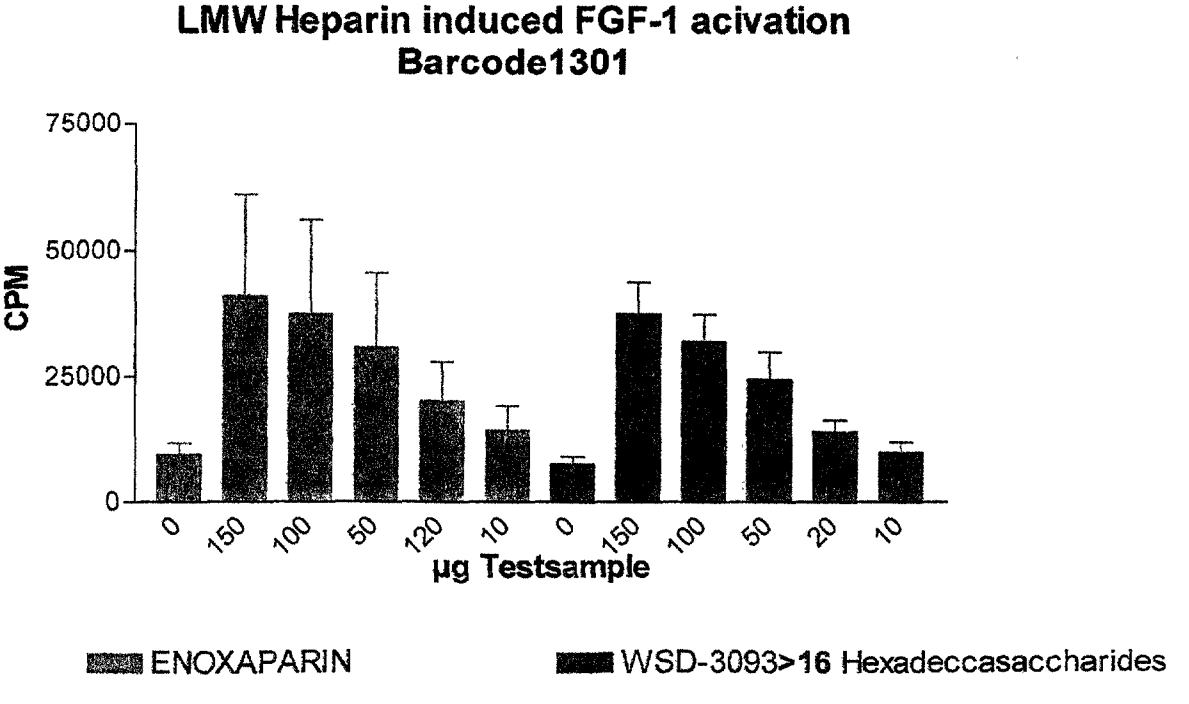


3.Comparison of WSD samples with Enoxaparin:

The WSD samples with shorter chain length: 6, 8, 10 and 12 are significantly less active compared to Enoxaparin (barcode 1296, 1297, 1298, 1299)



With higher chain length (<16, >16) no difference was observed (Barcode 1301)



Discussion.

The growth of BHK cells is dependent on the presence of aFGF. The growth promoting activity of aFGF can be increased in a dose dependent manner by addition of heparins. Under hypoxic conditions an upregulation of FGF receptors on endothelial cells is observed, in addition infiltrating granulocytes secrete FGFs and various other growth factors thus promoting angiogenesis. Therefore one can assume that angiogenesis can be promoted by heparins. We have tested this assumption in previous investigations using the well established Chorion allantois membrane (CAM) assay. We have found that low molecular heparins can promote angiogenesis compared to unfractionated heparin in a superior way. In particular in contrast to unfractionated heparin the application of higher doses of LMH does not result in suppression of angiogenesis.

The aim of the present study was the comparison of DIA versus WSD compounds of different chain length in the stimulation assay on BHK cells. From the results obtained all samples show to activate FGF-1. As can be seen from the results obtained on single doses on one plate a clear correlation between chain length and activity could be found. The assay is influenced by two important factors. The cell number and the length of time used for stimulation before the radioactive thymidine is added. If the stimulating substance is very active the cells divide very fast and can reach a plateau before the thymidine is added. The same observation is obtained if the cell number is too high. In both cases the values obtained are too low. In addition the amount of drug in relation to the cell number is of importance. Too low concentrations will not sufficiently stimulate the cells. We have therefore performed the assay testing all compounds on one plate with two different cell concentrations $4x10^4$ and $8x10^4$. As can be seen from the two assays at the 150µ dose (barcode 1405 and 1410) the amount of radioactivity is increased at the higher cell numbers; therefore, at the lower cell number the concentration of the stimulating substance is still in the optimal range. From both assays comparable results have been obtained. In summary we conclude that a clear positive correlation between chain length and activity is present. At the lower chain length, 6-10, there is a tendency towards higher activity of the WSD compounds which is reversed towards the DIA compounds at higher chain length. The observation that a decrease of activity is seen with the <Hexadecasaccharides samples is difficult to explain. We can exclude any weight mistakes because the amount of substance per vial had been indicated by the sponsor.

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I hereby certify that the experimental studies described and the analyses presented in this report were conducted by me and/or under my supervision.

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