

# APPENDIX D

**Regulation of FGF 2 mediated cell proliferation by Enoxaparin fractions or  
isolated specific oligosaccharides.**

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## Introduction

Cardiac infarction, cerebrovascular accidents and "smoker's leg" resulting from arteriosclerosis are among the commonest causes of death in the western industrial countries (5-10 cardiac infarctions per 1000 head of the population). However, biochemical and molecular biological research into the angiogenetic growth factors - particularly VEGF and FGF (Gospodarowicz et al., 1984) - has given rise to the hope that that these factors will show themselves to be effective for the treatment and prophylaxis of diseases caused by arteriosclerosis (Simons et al., 1996; Slavin, 1995). The proteins of the fibroblast growth factor (FGF) family are extremely efficient mitogens which can guide angiogenesis during the stages of development and growth (Klein et al., 1997; Schaper et al., 1996). Even in adult organisms suffering from ischemia the activity of angiogenetic factors can return (Sharma et al., 1992; Shweiki et al., 1992). This means that under certain circumstances the growth of collaterals can be induced, even to the extent that they compensate for the total occlusion of a main vessel. Various research groups have been able to show in appropriate animal experiments that exogenous administration of FGF to the ischemic area can increase angiogenesis in the heart or in peripheral regions of the body (Baffour et al., 1992; Battler et al., 1993; Giordano et al., 1996; Harada et al., 1994; Klein et al., 1997; Lazarous et al., 1995; Lazarous et al., 1996; Yanagisawa-Miwa et al., 1992).

The treatment of ischemic heart patients with bFGF was first carried out by our group (Schumacher et al., 1998). In a clinical trial, 20 patients with three-vessel coronary disease received bFGF by injection close to the vessel after the completion of an internal mammary artery (IMA) /left anterior descending coronary artery (LAD) bypass operation. In all the treated patients, but not in the 20 controls, a capillary network sprouting from the proximal part of the coronary artery could be shown to have bypassed the stenoses and rejoined the distal parts of the vessel after 12 weeks. As compared with the control patients, a three-fold increase of contrast medium accumulation was measured in the perivascular region of the LAD.

FGFs represent a family of over 20 members, regulating the growth, differentiation, survival and migration of a wide variety of cell types. In this family, FGF 2 (basic FGF) is a heparin-binding, 18KDa peptide, best known for its angiogenic, neurotropic, and mesoderm inducing effects.

In 1985, several authors have shown that Heparin strongly potentiates the growth-promoting activity of "endothelial growth factor" which is a form of FGF1-(acid FGF) and stimulates its angiogenic activity. In 1986, D. Gospodarowicz et al described the interactions of FGFs and Heparins. They also demonstrated that although FGF 2-heparin binding domain seems to be homologous to that of FGF 1, the growth-promoting activity of FGF 2 on human endothelial cells was inhibited by heparin ( Gospodarowicz D et al. )

Recent works by Khorana A.A. et al have compared the effect of Unfractionated Heparin , LMWHs (Enoxaparin, Tinzaparin, Dalteparin), Pentasaccharide (Fondaparinux) , and Oligosaccharides (Tetra , Octasaccharides) on human vascular endothelial cell (HUVEC) proliferation. They found that : (i) FGF 2 increased HUVEC proliferation (ii) Heparin and fractions 3000 and 6000 daltons inhibit FGF 2 dependent proliferation (particularly fraction 6000 daltons). (iii) Tetrasaccharide , Octasaccharide and Pentasaccharide (Fondaparinux) have no inhibitory action. When measuring the formation of capillary-like tube structures (cells cultured in Matrigel) in the presence of FGF 2 , they found that Heparin had no effect on tube formation, whereas the 3 commercial LMWHs (Enoxaparin, Tinzaparin, Dalteparin). had inhibitory action. So, Heparins have different effects on FGF 2 induced cell proliferation, partially dependent on molecular weight and may be other parameters. The authors concluded that heparin inhibition of endothelial cell proliferation and organization requires a chain length of >8 saccharide units, with maximal inhibition at Mr of 6kDa. This Mr dependence differs from that required for anticoagulant activity.

Previous studies (B.U. von Specht et al) have shown that Enoxaparin enhanced FGF 1 induced proliferation of the baby hamster kidney cell line BHK-21 . ( BHK cells have been shown to respond to both FGF 1 and FGF 2 ). In addition, this cell type is sensitive to heparin antiproliferative activity when grown in serum supplemented medium. Also, previous studies have shown that the interaction of Enoxaparin with FGF 1 is sensitive to structure variations such as the presence of a 1,6 anhydro ring. The stimulating effect of Enoxaparin on FGF 1 has also been confirmed in experimental models of angiogenesis such as the Chorio-allantoic membrane model.

According to the fact that FGF 2 is sensitive to heparin variations in molecular weight, saccharides chain length, and may be other parameters , FGF 2 can be considered as an attractive target for differentiation studies. .

The aim of the present study has been to compare the effect of series of compounds , fractions or oligosaccharides specifically present in Enoxaparin, on FGF 2 induced BHK cell proliferation , in vitro.

## 2. Materials and Methods

### FGF-2

Recombinant human basic fibroblast growth factor 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 trypsinized 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  $\mu$ l 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<sub>2</sub> incubator at 37°C with a humid atmosphere and 5% CO<sub>2</sub>.
- 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  $\mu$ l 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  $\mu$ l fresh stimulus medium is added to each well.
  - 20  $\mu$ l of concentrated solution in various dilutions is added to the medium as a test substance.

For the eight-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-<sup>3</sup>H] thymidine (Amersham, 5 mCi/5 ml, TRK 300) with a final concentration of 1  $\mu$ Ci 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 FilterMate™ Cell Harvester through a UniFilter™ Plate of 96 format with an integrated GF/C-Filter and discarded.
- 100  $\mu$ l 0.05% trypsin / 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 samples are simultaneously completely harvested with FilterMate™ Cell Harvester onto the UniFilter™ Plate. The plates are so constructed that each filter within a well is completely separated from the neighboring filters. The UniFilter™ Plate is washed 5 times with deionized water and then dried at 55°C for at least 30 minutes.
- 30  $\mu$ l of the scintillation fluid MICROSCINT™ 20 (Packard, 11, Cat.-No.6013621) is placed into each well and counted with Topcount NXT™.

As a result from extensive pre testing, cells were grown at a constant concentration of 1,4 ng/ml FGF-2 and between 0,1-500  $\mu$ g/ml of the test heparin. The results of one assay with heparin assay is shown in FIG-1. Each concentration values represent mean values of eight single values.

**Barcode 1635**  
**Heparin induced FGF-2 Inactivation**  
 cells  $1,5 \times 10^4$ /ml  
 (Control assay 48)

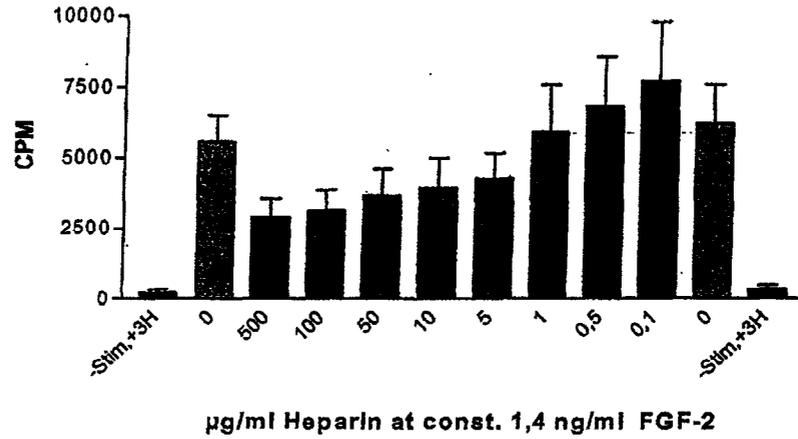


FIG-1

**Analysing scheme**

|           |          |          |          |          |          |          |          |          |          |           |           |           |
|-----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|-----------|-----------|-----------|
| <b>A1</b> | <b>2</b> | <b>3</b> | <b>4</b> | <b>5</b> | <b>6</b> | <b>7</b> | <b>8</b> | <b>9</b> | <b>9</b> | <b>10</b> | <b>11</b> | <b>12</b> |
| <b>1B</b> |          |          |          |          |          |          |          |          |          |           |           |           |
| <b>C</b>  |          |          |          |          |          |          |          |          |          |           |           |           |
| <b>D</b>  |          |          |          |          |          |          |          |          |          |           |           |           |
| <b>E</b>  |          |          |          |          |          |          |          |          |          |           |           |           |
| <b>F</b>  |          |          |          |          |          |          |          |          |          |           |           |           |
| <b>G</b>  |          |          |          |          |          |          |          |          |          |           |           |           |
| <b>H</b>  |          |          |          |          |          |          |          |          |          |           |           |           |

A1-H1 control only cells no FGF

A2-H2 control cells +1,4ng/ml FGF-2, no heparin

A3-H3-A11-H11 Increasing values of heparin at constant amount of 1,4ng FGF-2

A12-H12 control cells +1,4ng/ml FGF-2, no heparin

**Experimental groups****Series 1:**

Dose response curves for each test sample. According to FIG 1. One test sample is analyzed on a single plate in various concentrations (500-0,1 µg/ml). A control plate with heparin is included to each assay.

**Series 2**

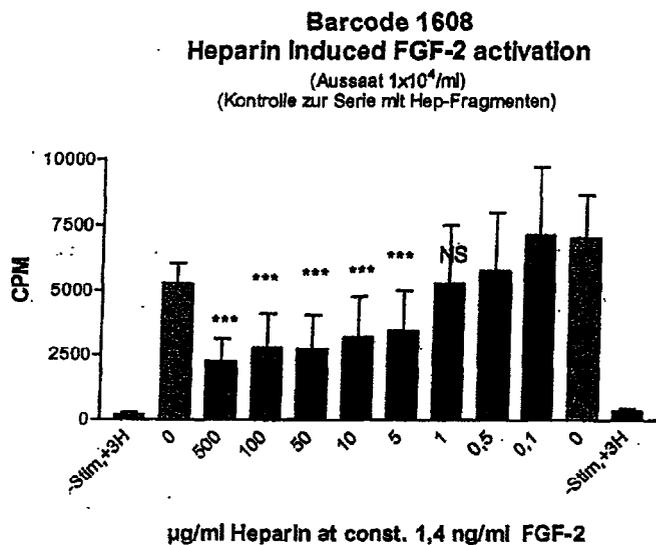
Comparison of test samples (group 1, sample 1-7; versus group 2, samples 8-14; group 3 samples 15-17) on one plate at constant concentration of HEP fragment

**Statistics:**

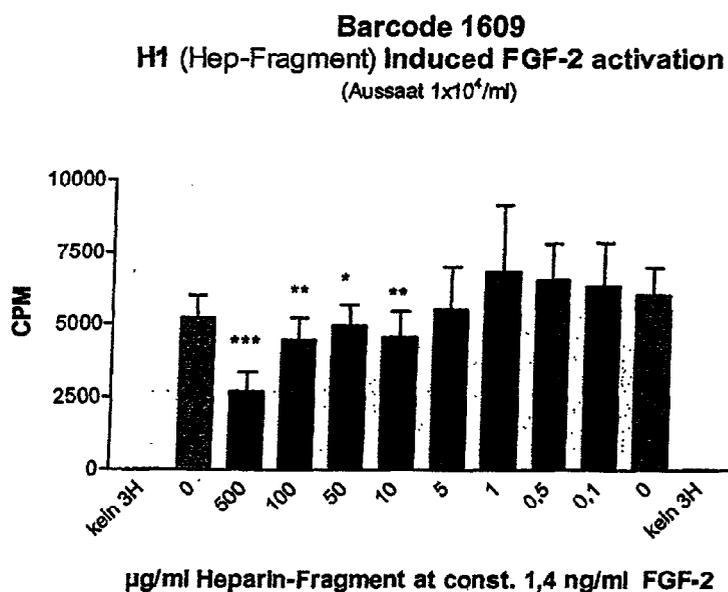
Results are expressed as mean +/- SE. Statistical significance of differences in means between groups have been determined by using a two tailed students t test. A value of  $p < 0,05$  has been considered significant. All experiments have been reproduced at least twice, in eightfold values. Statistics have been calculated with the computer program Prism™. The original data can be studied by double clicking on the individual graphs on the assay sheets if the Prism program is installed on the computer

## Results part 1 compounds HEP 1-7

Assays 43-44 (appendix)



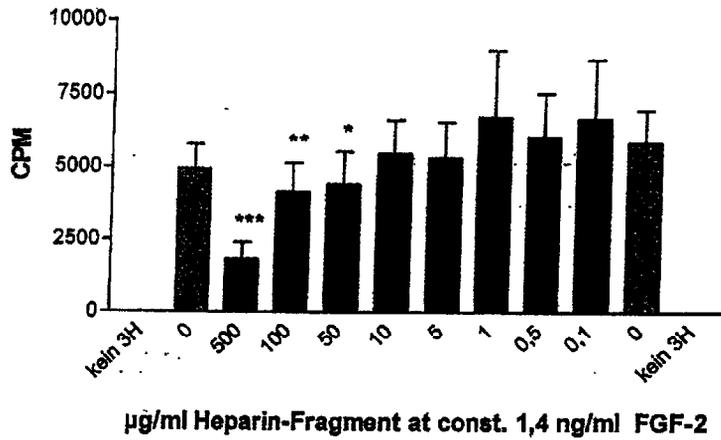
Heparin induces a dose dependent inhibition of proliferation of BHK cells which is significant in a dose range between 500-5µg/ml



\* P value 0,0239; \*\* P value 0,0026; \*\*\* P value <0,0001

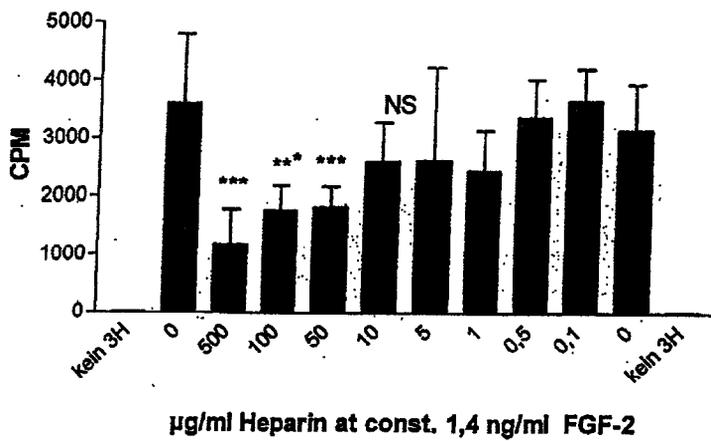
Hep 1: Inhibition is significant in the dose range 500-10µg/ml. At lower doses 0,1-1µg/ml no significant effect can be observed

**Barcode 1610**  
**H2 (Hep-Fragment) induced FGF-2 activation**  
 (Aussaat  $1 \times 10^4$ /ml)



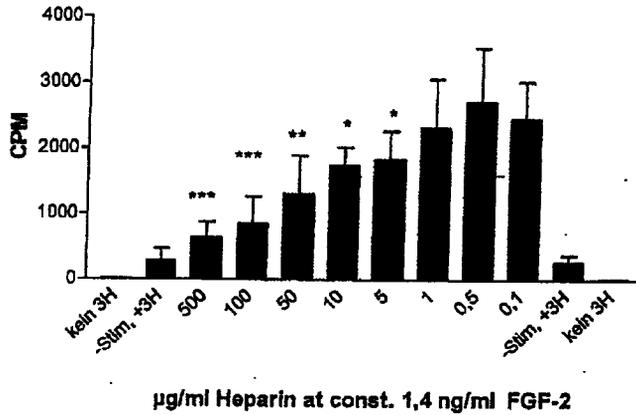
**Hep2: significant inhibition only between 500-50µg. No effect between 10-0,1 µg/ml dose**

**Barcode 1603**  
**H3 (Hep-Fragment) induced FGF-2 activation**  
 (Aussaat  $1 \times 10^4$ /ml)



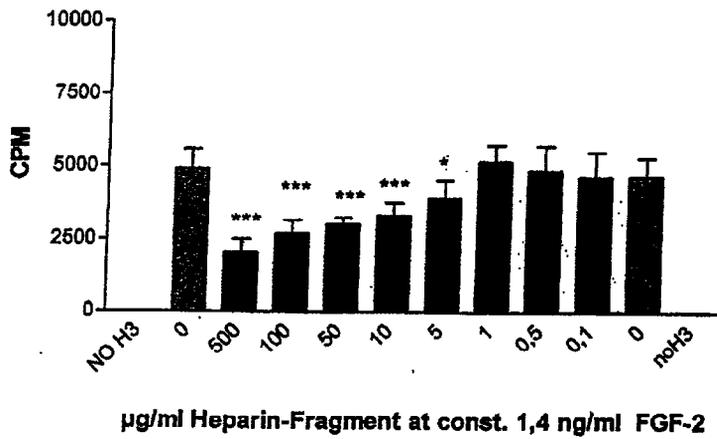
**Hep 3: Inhibition only 500-50µg dose. No effect 0,1-10 µg dose**

**Barcode 1604**  
**H4 (Hep-Fragment) induced FGF-2 inactivation**  
 (cells  $1 \times 10^4$ /ml)



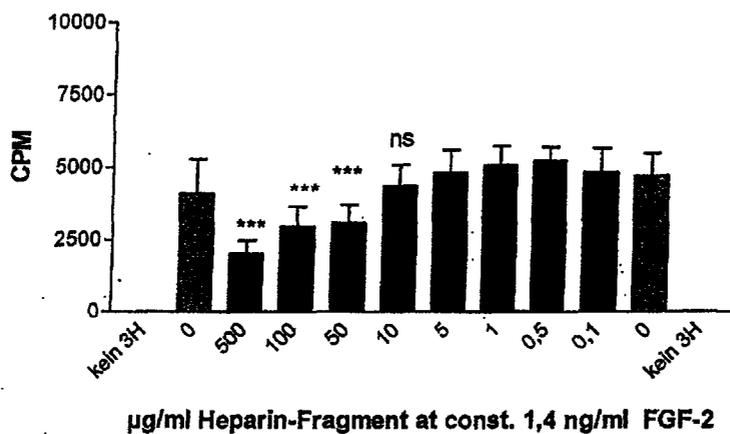
**Hep 4: Dose dependent inhibition between 500-5 µg dose. Similar inhibition pattern like Heparin**

**Barcode 1613**  
**H5 (Hep-Fragment) induced FGF-2 inactivation**  
 (cells  $1 \times 10^4$ /ml)



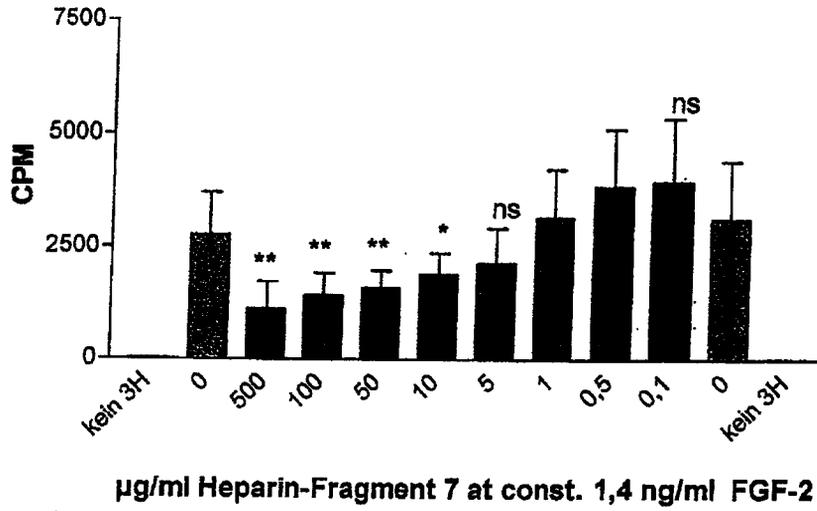
**Hep 5: Dose dependent inhibition between 500-5 µg. No effect between 0,1-1 µg dose**

**Barcode 1614**  
**H6 (Hep-Fragment) induced FGF-2 inactivation**  
(cells  $1 \times 10^4$ /ml)



**Hep 6: inhibition between 500-50µg dose. No effect between 0,1-5 µg/ml dose**

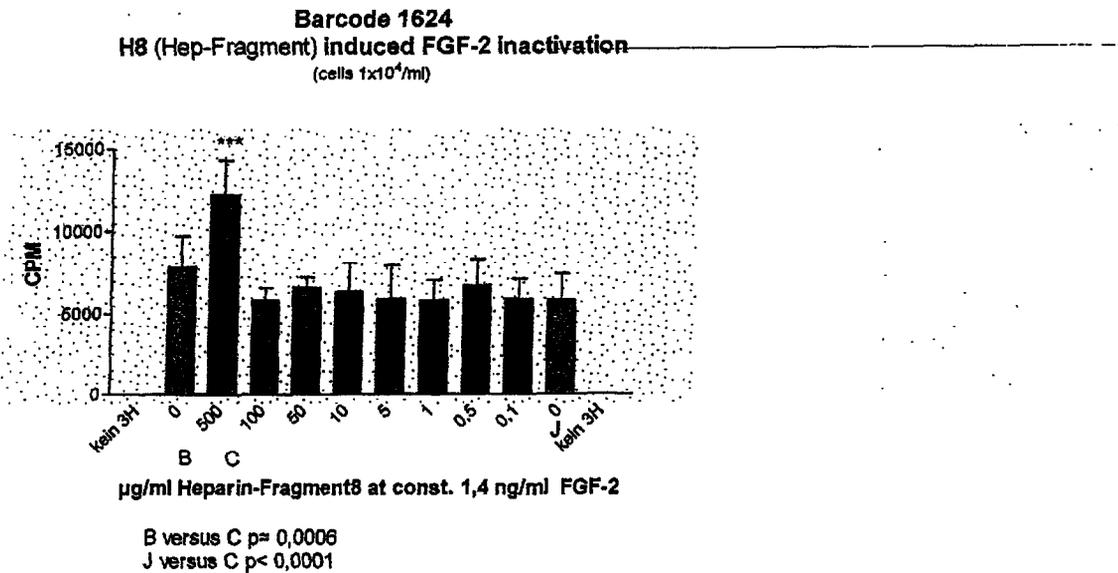
**Barcode 1642**  
**Hep7 (Hep-Fragment) induced FGF-2**  
**inactivation**  
(Aussaat  $1,5 \times 10^4$ /ml)



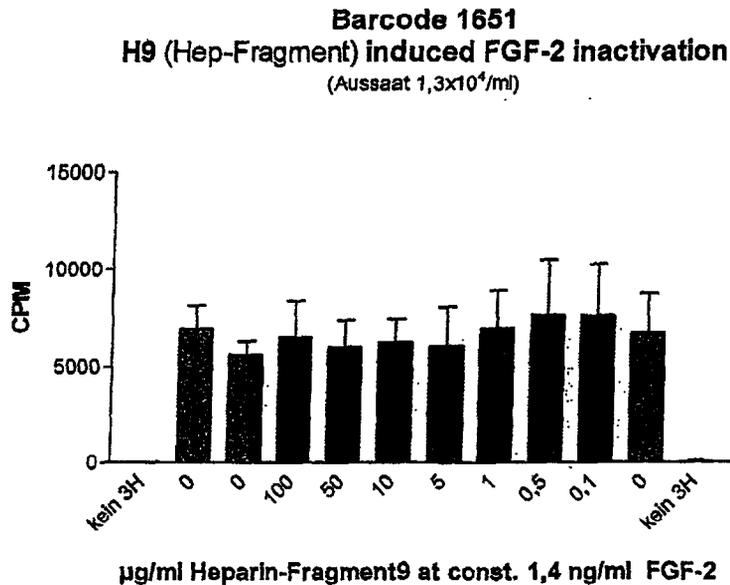
**Hep 7: Inhibition between 500-10µg dose. Effects at lower doses are not significant.**

## Results part 2 compounds 8-14

Assays 46,49,50 (appendix)

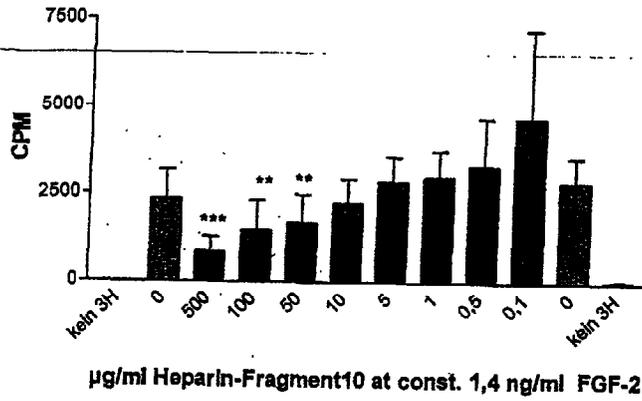


**Hep 8: No inhibitory effect between 0,1-100µgdose range Activation of proliferation at the 500µg dose.**



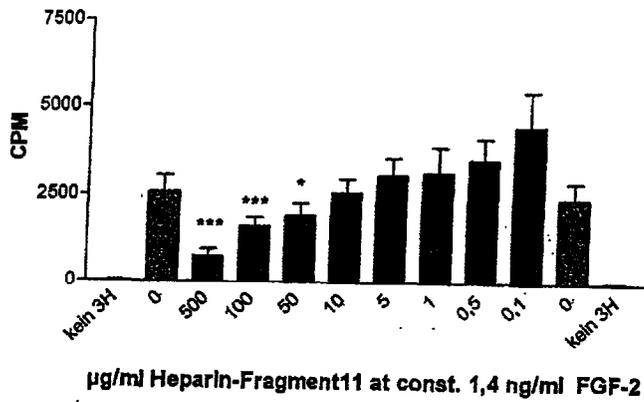
**Hep 9: No inhibitory effect between 0,1-100 µg dose**

**Barcode 1645**  
**H10 (Hep-Fragment) induced FGF-2**  
**Inactivation**  
 (Ausssaat  $1,3 \times 10^4$ /ml)



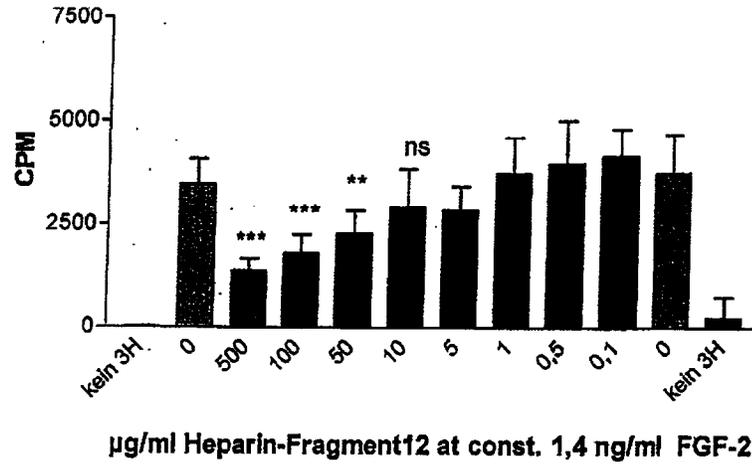
**Hep10: Inhibition between 500-50 µg**

**Barcode 1646**  
**H11 (Hep-Fragment) induced FGF-2**  
**inactivation**  
 (Ausssaat  $1,3 \times 10^4$ /ml)



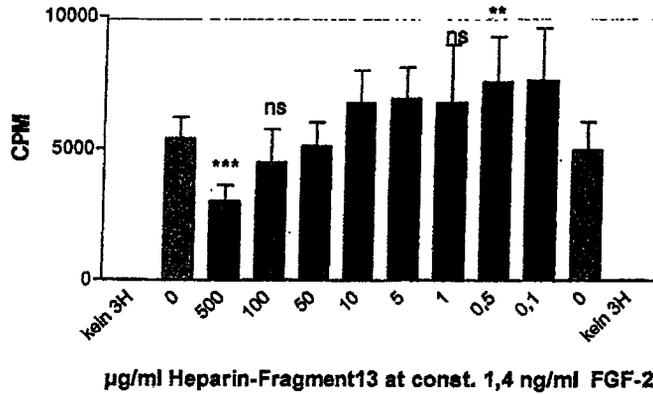
**Hepp11: Inhibition between 500-50 µg**

**Barcode 1647**  
**Hep12 (Hep-Fragment) induced FGF-2**  
**inactivation**  
(cells  $1,5 \times 10^4$ /ml)



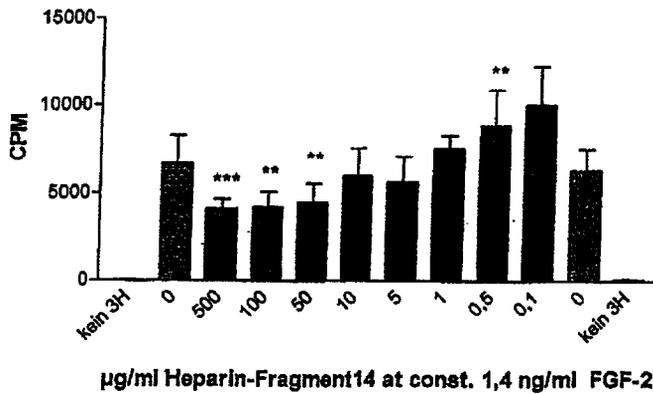
**Hep12: Inhibition between 500-50  $\mu$ g**

**Barcode 1636**  
**H13 (Hep-Fragment) induced FGF-2**  
**inactivation**  
 (Aussaat  $1,5 \times 10^4$ /ml)



**Hep13: Significant activation at low dose (0,5-0,1 µg/ml). Trend (not significant) towards activation at medium dose(1-10µg). Inhibition only at high dose ( 500 µg)**

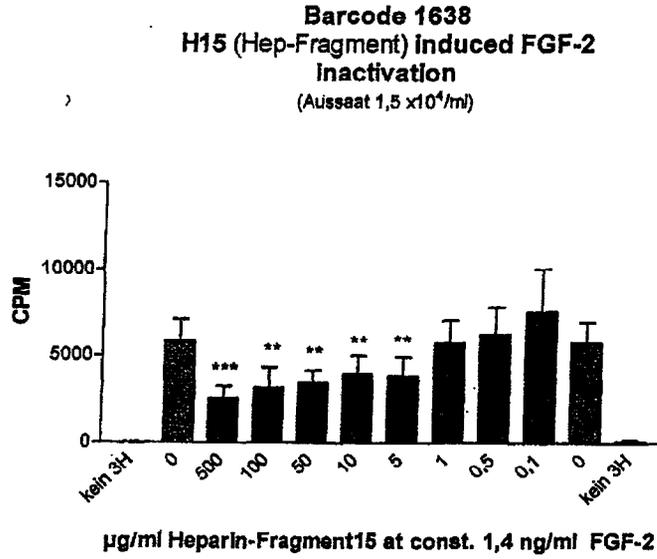
**Barcode 1637**  
**H14 (Hep-Fragment) induced FGF-2**  
**inactivation**  
 (Aussaat  $1,5 \times 10^4$ /ml)



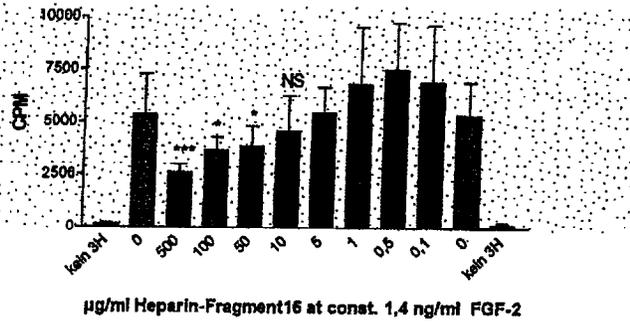
**Hep14: Significant activation at low dose (0,5-0,1 µg/ml). No effect at medium dose(1-10µg). Inhibition only at high dose ( 500- 50µg)**

**Results part 3 compounds 15-17**

ASSAYS 47, 48 appendix

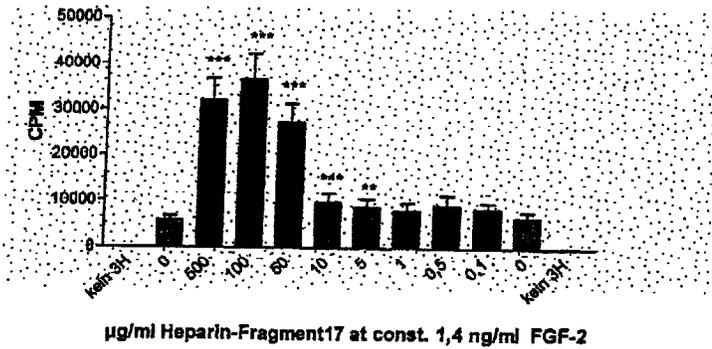
**Hep 15: significant inhibition 5-500 $\mu\text{g/ml}$**

**Barcode 1639**  
**H16 (Hep-Fragment) Induced FGF-2**  
**inactivation**  
 (Ausssaat  $1,5 \times 10^4/m$ )



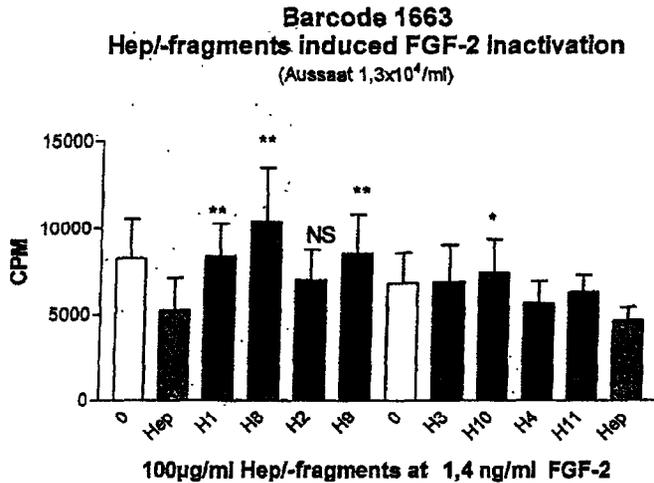
**Hep 16 inhibition at high dose (500-50 µg/ml). Trend towards activation (not significant) at low dose**

**Barcode 1640**  
**H17 (Hep-Fragment) Induced FGF-2**  
**activation**  
 (Ausssaat  $1,5 \times 10^4/m$ )



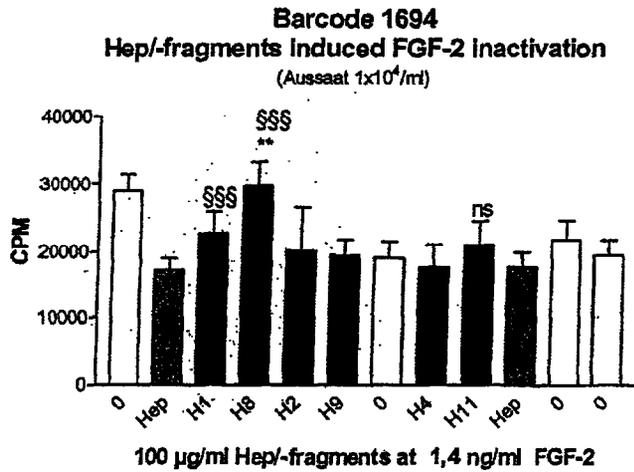
**HEP17 High activation between 500-5 µg/ml dose**

**Results part 4 comparison of individual HEP compounds, Appendix assays 55,56**



\* Comparison versus heparin

In this graph we have analyzed the effect of 100µg HEP compound versus the same dose of heparin. At the 100µg dose HEP 1, Hep 8, Hep9 and HEP 10 treated cells show a higher proliferation rate (less inhibition) compared to the same dose of heparin

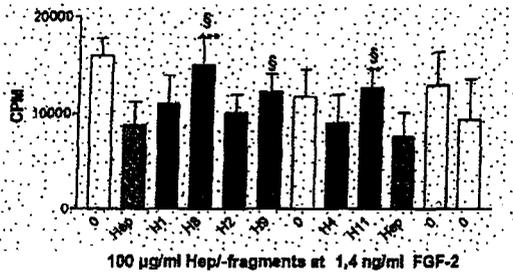


\*\* HEP1 v HEP 8    ns HEP 4 v HEP 11  
 \$\$\$ HEP v HEP 1    \$\$\$ HEP v HEP 8

This graph analyzes the differences of individual HEP compounds. Cells treated with the 100µg dose HEP8 are significantly more active compared to cells treated with the same dose of HEP 1

No difference between HEP2 and HEP 9, was measured. The difference between HEP 4 and HEP 11 is not significant in this assay.

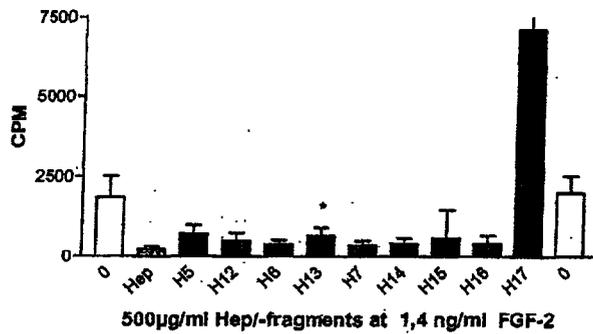
**Barcode 1686**  
**Hep/-fragments induced FGF-2 inactivation**  
 (cells  $1 \times 10^4$ /ml)



§ p = 0,0128 H1 versus H8  
 § p = 0,038 H2 versus H8  
 § p = 0,0128 H4 versus H11  
 \*\*\* p = 0,0002 Heparin versus H8  
 \*\* p = 0,0043 H 11 versus Heparin

This graph analyzes the difference of individual HEP compounds.  
 Cells treated with a 100µg dose of HEP 8 are significantly less inhibited compared to HEP 1  
 Cells treated with a 100µg dose of HEP 9 are significantly less inhibited compared to HEP 2  
 Cells treated with a 100µg dose of HEP 11 are significantly less inhibited compared to HEP 4

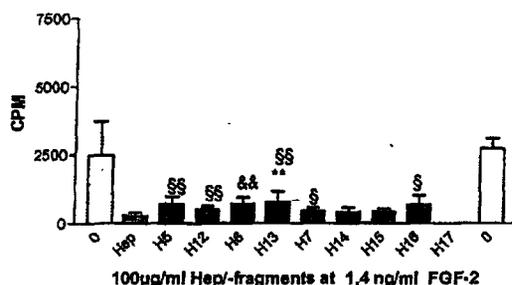
**Barcode 1670**  
**Hep/-fragments induced FGF-2 inactivation**  
 (Ausset  $1 \times 10^4$ /ml)



H5 versus H12 ns  
 H6 versus H13 p=0,0214  
 H7 versus H14 ns

This graph analyzes the influence of individual HEP compounds on the inhibition of the proliferation rate of BHK cells at a 500µg/ml dose  
 HEP 5 compared to HEP 12 no significant difference  
 HEP 6 compared to HEP 13 significant difference  
 HEP 7 compared to HEP 14 no significant difference

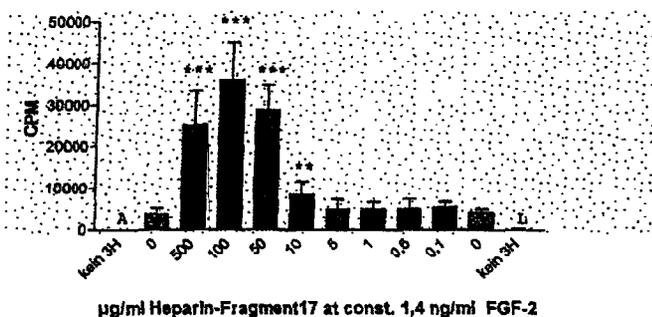
**Barcode 1671**  
**Hep-fragments induced FGF-2 inactivation**  
 (Aussaat  $1 \times 10^4$ /ml) H17 nicht dargestellt ( $\geq 7500$  CPM)



\*\* H13 versus control (no heparin) H7 versus H14 not significant  
 H5 versus H12 not significant  
 \$\$\$ H 13 versus Heparin H6 versus H13 ns p= 0.5931  
 HEP 5 versus HEP12 ns p= 0,1351

This graph analyzes the influence of individual HEP compounds on the proliferation rate of BHK cells at a 100 µg/ml dose. All HEP compounds (5,12,6,13,7,14,15,16) inhibit significantly the proliferation rate of BHK cells compared to control cells without heparin (open bars). Compared to Standard heparin HEPs 5, 12, 6,13,7 and 16 show a significant higher proliferation rate. No significant differences can be measured between HEP 5 and HEP 12; HEP 6 and HEP 13, HEP 7 and HEP 14 at the 100 µg/ml dose

**Barcode 1634**  
**H17 (Hep-Fragment) induced FGF-2 inactivation**  
 (Aussaat  $1,5 \times 10^4$ /ml)



This graph analyses the influence of different doses of HEP 17 on the proliferation rate of BHK cells. Compared to control cells (no heparin, Columns B and K), HEP 17 induces a highly significant increase of proliferation in the dose range between 10-500 µg/ml

**Comparison of individual HEP compounds at the 100µg dose. Proliferation rates of BHK-cells**

|               |               |            |                |                  |                                |                  |
|---------------|---------------|------------|----------------|------------------|--------------------------------|------------------|
| HEP1          | HEP2          | HEP3       | HEP4           | HEP5             | HEP6                           | HEP7             |
| HEP8          | HEP9          | HEP10      | HEP11          | HEP12            | HEP13                          | HEP14            |
| HEP8><br>HEP1 | HEP9><br>HEP2 | HEP3=HEP10 | HEP11><br>HEP4 | No<br>difference | HEP13>HEP6<br>At 500µg<br>dose | No<br>difference |

**Results:** Beside Substance HEP 17, significant effects of HEP fragments on FGF-2 mediated cell proliferation could be measured only at high doses (500-50 µg/ml).

**Activation** of proliferation compared to cells stimulated with FGF-2 only was observed with HEP 17. A significant increase was measured at doses between 10-500µg. Activation was also observed but only at the high dose of 500µg with HEP 8.

**Inhibition** of cell proliferation to about 30% of maximal proliferation rates was measured at the 500-50 µg dose range with all HEP fragments beside HEP 8 (activation 500µg); HEP 9 (no significant effect) and HEP17 (activation at doses between 10-500µg)

**Comparison to standard Heparin:** HEPs 1,8,9,10,11,13 showed significant higher stimulation rates (less inactivation) at the 100µg dose compared to the same dose of heparin.

**Direct comparison of HEP fragments (100µg dose):** Inhibition of proliferation HEP1>HEP8; HEP2>HEP9; HEP4>HEP 11.

No significant difference: HEP3 versus HEP10; HEP5 versus HEP12; HEP 6 versus HEP 13 (at 500µg HEP 6 more inhibition) and HEP 7 versus HEP 14.

**Discussion:**

Cell proliferation assays show the problem of relative high standard deviations. Therefore 8 fold values for each measurement were analysed and each experiment reproduced 2-3 times.

In the first set of experiments samples were analysed at different concentrations (500-0,1µg) each sample on one plate and compared to heparin at the same dose range. Heparin as a control inhibited cell proliferation from 7500 cpm to 2500cpm. This inhibition rate of about 66% could be observed in all assays. The assay is able to respond to minimal doses of 5µg/ml of heparin with significant changes of proliferation rates. The non significant activation effect seen at very low doses is due to an unspecific stabilization effect of heparin to the highly diluted FGF-2.

It was the aim of the study to investigate whether structural differences between to groups of HEP fragments (Group1 HEP 1-7, Group 2 HEP 8-14) would have consequences on the rate of inhibition of the proliferation of BHK cells. The inter action of FGF-2 with heparins of various chain length and consequently a reduced ability to promote the growth of endothelial cells was published recently by Khorana A.A. et al. From our results we are able to conclude that the substances HEP8 - HEP14 inhibit the FGF-2 dependent growth of BHK cells to a lesser extend. HEP 8 shows no significant inhibition over the whole dose range and activation at the 500µg dose. Statistical significant differences between HEP fragments could be measured only at high (100-500µg) per ml doses of HEP fragment.

Additional control substances (HEP 15,16,17) were analyzed in a blinded manner. The assay was clearly able to identify the activating potency of HEP 17 and to discriminate between HEP 15 and HEP 16.(Heparin and Enoxaparin).

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