

APPENDIX G

Effect of Enoxaparin and Novel Polysaccharides
on Proliferation of Vascular
Smooth Muscle Cells

**Effect Of Enoxaparin And Novel
Polysaccharides On
Proliferation Of Vascular Smooth Muscle
Cells**

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Morphology and *Cell Biology of Diabetes Laboratories

Baker Heart Research Institute

Research Report to

Dr Andre Uzan

Aventis

Centre de Recherche de Vitry

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

PRINT NAME

Rodney Dilley

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DATE

27.1.04

September 2003



Baker
Heart Research Institute

Aim: To determine the effects of novel polysaccharide compounds and the low molecular weight heparin compound enoxaparin on cell proliferation in human vascular smooth muscle cells in vitro.

Cell culture experiments –

Smooth muscle cells were isolated from explants of human internal mammary artery samples and grown in Dulbecco's modified Eagles medium containing 10% fetal calf serum. The experiments were performed on cells in subpassage 15 or 16.

Analysis of cell growth

Cells were plated at low density (5,000 cells/cm²) in plastic culture dishes (7cm²) and allowed to attach for 1 day, then cells were growth-arrested by addition of 0.1% serum for 3 days. Growth was assessed in medium containing 5% fetal calf serum which was added for 3 days and then cells were released from the dish with trypsin-EDTA for 2 minutes and suspended in Dulbeccos phosphate buffered saline for counting. Total cell number in each dish was measured using a Coulter counter. Growth was calculated as cell number at the end of 5% serum treatment (day 7) minus cell number at the end of 0.1% serum treatment (day 4). These data were compared against cell number at the beginning of treatment (day 4) to ensure similar growth rates in cultures.

Dose-dependent growth inhibition

For a dose-response analysis the effect of test substances at one of five different concentrations (1, 3, 10, 30, 100 µg/ml) on cell growth was determined. For each experimental compound growth inhibition during three days treatment was calculated (growth in 5% serum minus growth in 5% serum plus drug) and also compared against growth inhibition by enoxaparin treatment (at 100 µg/ml) in the same experiment. Experiments were performed in duplicate for each concentration, on two separate occasions.

Data analysis and reporting

The data were plotted against the control and compared by t test against enoxaparin treatment using Excel software.

RESULTS

Cell Growth in 5% serum

In this assay vascular smooth muscle cells stimulated from growth arrest with 5% serum responded with on average a 6.4 fold increase in cell number over 3 days and a 4.6 fold increase over cells grown in 0.1% serum ($P < 0.05$, Figure 1) over the same period, which increased in number only 1.5 fold (n.s., $P = 0.23$).

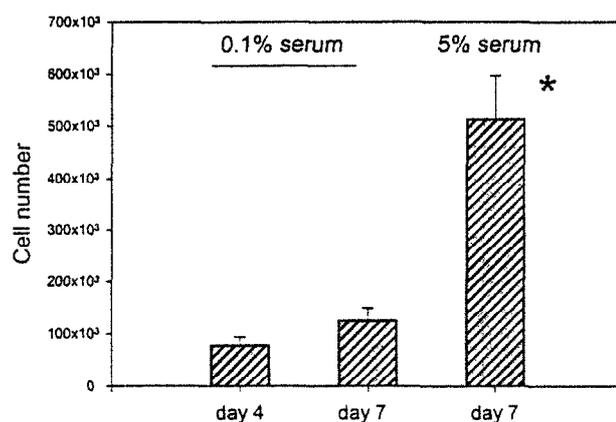


Figure 1. Cell number in cultures after growth in 0.1% serum (day 4 and 7) and after 3 days growth in 5% serum. (* $P < 0.05$ vs 0.1% serum day 4, day 7)

Growth inhibition by heparin and enoxaparin

Growth in this assay system was inhibited 26% by unfractionated heparin (Sigma grade 1A, 100 $\mu\text{g}/\text{ml}$) to only 74% of the growth seen in the absence of heparin. In a preliminary experiment, enoxaparin was seen to inhibit growth to a similar level (27%).

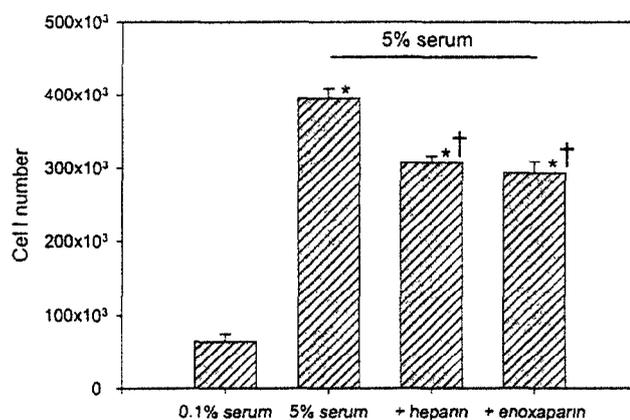


Figure 2. Growth inhibition by unfractionated heparin and enoxaparin after 7 days in culture (* $P < 0.05$ vs 0.1% serum, † $P < 0.05$ vs 5% serum)

Inhibition by enoxaparin was found to be dose-dependent with an IC_{50} between 3 and 10 $\mu\text{g}/\text{ml}$. A positive control group at 100 $\mu\text{g}/\text{ml}$ enoxaparin was included (in

quadruplicate) in every experiment performed, and the growth inhibition observed in these 5 experiments was on average $31 \pm 2.8\%$ (mean \pm SEM).

Enoxaparin and polysaccharide compounds

The twelve compounds provided by Aventis were in two groups, 6 of which were WSD3093 compounds and 6 DIA2844. These compounds were paired, one each of WSD3093 and DIA2844 with the following structures:

1. Hexasaccharides
2. Octasaccharides
3. Decasaccharides
4. Dodecasaccharides
5. <Hexadecasaccharides
6. \geq Hexadecasaccharides

We tested these compounds in a dose response over two orders of magnitude ($1 \mu\text{g/ml}$ to $100 \mu\text{g/ml}$) and found heterogeneous effects on SMC growth.

Most produced growth inhibition at $100 \mu\text{g/ml}$ concentration (Table 1), ranging from a non-significant 3% up to 28% inhibition for DIA2844 \geq hexadecasaccharides, which was equivalent to the magnitude of inhibition by enoxaparin ($P = 0.46$) at equal mass in our assay system.

Table 1 Growth inhibition at $100 \mu\text{g/ml}$ concentration of test compound. Percent inhibition of growth in 5% serum.

	WSD3093	DIA2844
Hexasaccharides	$9 \pm 3^*$	$16 \pm 2^*$
Octasaccharides	$18 \pm 2^*$	$21 \pm 2^*$
Decasaccharides	$11 \pm 2^*$	$15 \pm 5^*$
Dodecasaccharides	$26 \pm 11^*$	$10 \pm 6^*$
<Hexadecasaccharides	$19 \pm 5^*$	3 ± 7
\geq Hexadecasaccharides	$26 \pm 11^*$	$28 \pm 4^*$

* = $P < 0.05$ for cell number in test compound at $100 \mu\text{g/ml}$ vs 5% serum control

The greatest inhibition was found with the largest WSD3093 and DIA2844 structures, the \geq hexadecasaccharides, however there was not a distinctive pattern according to size as DIA2844 octasaccharides inhibited growth (21%) almost to the same degree as the \geq hexadecasaccharides compounds, which were presumably at least twice their size. The <hexadecasaccharides did not significantly inhibit growth.

When compounds were compared pairwise, there were no significant differences in growth inhibition between WSD3093 and DIA2844 at any of the size ranges identified (Table 2).

Table 2 Results of statistical analysis of percent growth inhibition by WSD3093 compounds vs DIA2844 compounds with same structure

	P for WSD3093 v DIA2844
Hexasaccharides	P>0.05 (0.114)
Octasaccharides	P>0.05 (0.357)
Decasaccharides	P>0.05 (0.606)
Dodecasaccharides	P>0.05 (0.081)
<Hexadecasaccharides	P>0.05 (0.203)
≧Hexadecasaccharides	P>0.05 (0.828)

Paired t test comparing WSD3093 v DIA2844 compounds at 100 µg/ml, n = 4

Dose dependence of growth inhibition

An apparent increase in growth inhibition with increasing concentration was observed for several of the compounds tested (Figure 3, 4). Some of both DIA2844 and WSD3093 compounds had apparent dose-dependent effects.

In order to test for a dose response relationship statistically we determined regression coefficients for each plot of percent growth inhibition (Sigmaplot 2002) over the full dose range (0.1-100 µg/ml). Regression curves are presented in Figures 3 and 4.

The statistical analysis allowed testing for a dose relationship according to the 'a' constant (slope factor) in the function $f=y_0+a*x(b+x)$. A significant slope ($P<0.05$) was observed for the WSD3093 compounds with the structures octasaccharides, <hexadecasaccharides and \geq hexadecasaccharides. For the DIA2844 compounds only the \geq hexadecasaccharides structure showed a statistically significant dose effect.

Figure 3
Test compound WSD3093 concentration vs inhibition curves after 3 days treatment of cells growing in DMEM with 5% serum. Individual points are plotted for four determinations at each concentration point.

WSD3093

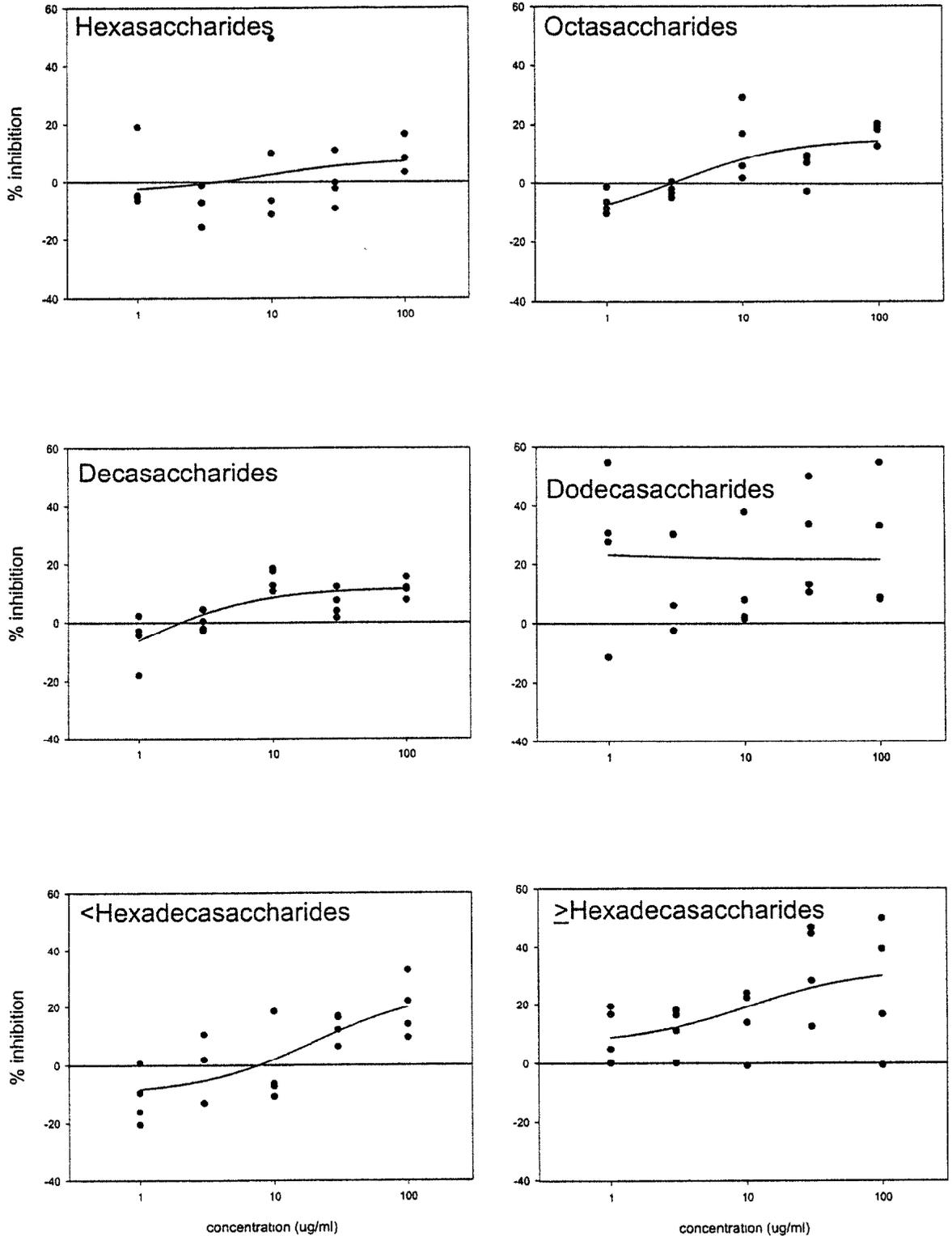
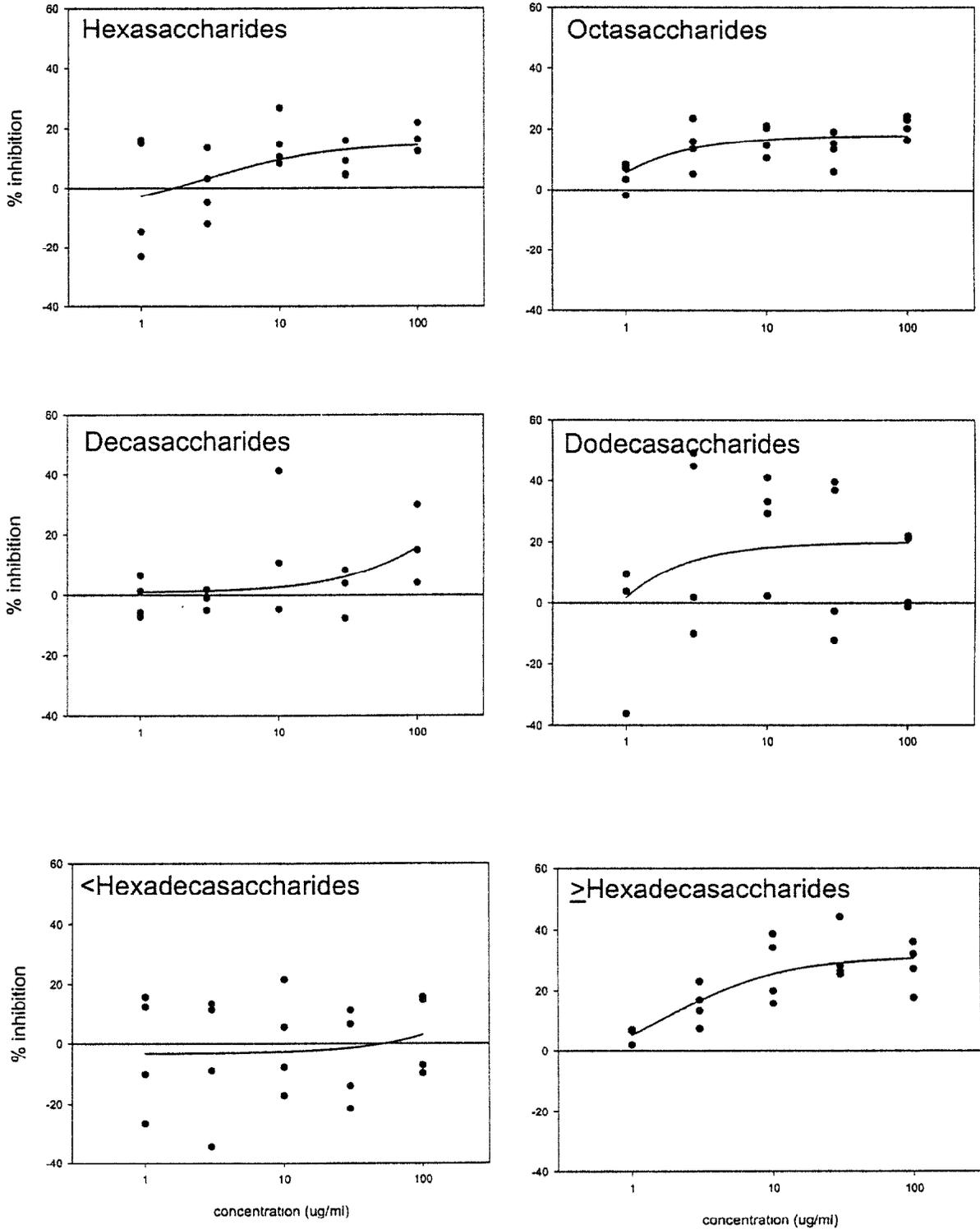


Figure 4

Test compound DIA2844 concentration vs inhibition curves after 3 days treatment of cells growing in DMEM with 5% serum. Individual points are plotted for four determinations at each concentration point.

DIA 2844



Supplement Report to
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PRINT NAME

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27.1.04

December 2003



Aim: To compare the vascular smooth muscle cell growth inhibitory effects of novel polysaccharide compounds: <hexasaccharides from WSD3093 and DIA2844.

Cell culture experiments –

Smooth muscle cells were isolated from explants of human internal mammary artery samples and grown in Dulbecco's modified Eagles medium containing 10% fetal calf serum. The experiments were performed on cells in subpassage 15 or 16.

Analysis of cell growth

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Data analysis and reporting

The data were plotted against the control and compared by t test against each other using Excel software. Curves were fitted and linear regression performed using Sigmaplot software.

RESULTS

Enoxaparin and polysaccharide compounds

The two compounds provided by Aventis for this specific comparison were both in the size range <hexadecasaccharides with WSD3093 and DIA2844 structural modifications.

We tested these compounds four times in a dose response over two orders of magnitude (1µg/ml to 100µg/ml) and found heterogeneous effects on SMC growth.

WSD3093 <Hexadecasaccharides produced growth inhibition at 100 µg/ml concentration (Table 1). The values for each culture ranged from a non-significant 6% up to 33% inhibition for WSD3093 <hexadecasaccharides at this concentration. DIA2844 also produced an apparent 16% inhibition on one occasion but this was not dose dependent (see below) and considered an artefact in this culture and mean values were much lower.

Table 1 Growth inhibition at 100 µg/ml concentration of test compound. Percent inhibition of growth in 5% serum.

	WSD3093	DIA2844
<Hexadecasaccharides	16 ± 3*	3 ± 3

* = P < 0.05 for cell number in test compound at 100 µg/ml vs 5% serum control

The greatest inhibition was found with the WSD3093 structures, mean inhibition values being approximately five-fold greater than those observed for DIA2844 (P=0.034, paired t test). The <hexadecasaccharides DIA2844 did not significantly inhibit growth.

Dose dependence of growth inhibition

An apparent increase in growth inhibition with increasing concentration was observed for WSD3093 compounds. There was no significant dose dependence for DIA 2844 compounds.

In order to test for a dose response relationship statistically we determined regression coefficients for each plot of percent growth inhibition (Sigmaplot 2002) over the full dose range (0.1-100 µg/ml). Regression curves are presented in Figures 3 and 4.

The statistical analysis allowed testing for a dose relationship according to the 'a' constant (slope factor) in the function $f=y_0+a*x(b+x)$. A significant slope ($P<0.05$) was observed for the WSD3093 <hexadecasaccharides compounds and not for DIA2844 compounds.

