



# APPENDIX H

*In Vitro* HIT Cross-reactivity of Chemical  
Synthetic Oligosaccharides

***In vitro* HIT cross-reactivity  
of chemical synthetic oligosaccharides**

**Study of fifty HIT sera  
in citrated human platelet rich plasma**

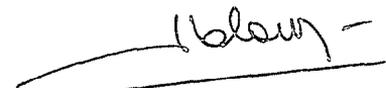
October 2003

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

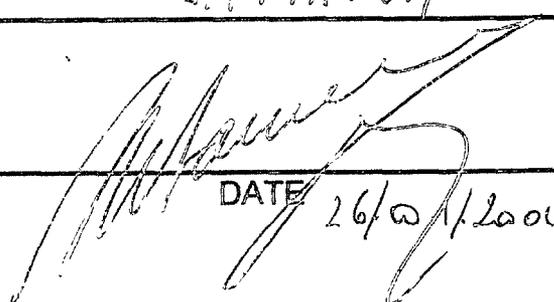
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## **I. BACKGROUND**

Heparin-Induced Thrombocytopenia (HIT) is the most severe complication of heparin therapy with a high risk of thrombosis (1). HIT results from formation of heparin-dependent antiplatelet antibodies whose major target is a macromolecular complex associating heparin and platelet factor 4 (PF4), a tetrameric cationic protein produced from platelet alpha granules. HIT is characterized by a delayed occurrence, resistance to heparin anticoagulant effect, abrupt fall of platelet count and a high vascular morbidity and mortality. Its incidence varies from 1 to 5% leading to an acquired hypercoagulable state requiring an alternative antithrombotic drug. The effect of HIT antibodies may be evidenced *in vitro* on normal selected platelets after the addition of unfractionated heparin (UFH; 0.5 and 1.0 anti-Xa IU/ml) according to the heparin induced platelet aggregation test (PAT) described by Fratantoni (2) and modified by our group (3). The occurrence of *in vitro* cross-reactivity may be due to the presence of immunoglobulin species reacting with glycosaminoglycan epitopes similar to those of heparin.

## **II. AIM OF THE STUDY**

The aim of this study is to determine the potential *in vitro* HIT cross-reactivity of various oligosaccharides at 3 different dosages using PAT. We will also use Pentasaccharide (Arixtra<sup>®</sup>) to compare their potency to provoke such a platelet response (4).

## **III. MATERIALS AND METHODS**

Citrated whole blood is obtained from healthy fasting donors whose platelets belonged to the class of "responders" in the presence of plasma from a HIT patient

and UFH. Platelet rich plasma (PRP, 400 to 500 G/l) is prepared after centrifugation to obtain a constant value for PAT analysis. Fifty frozen HIT positive plasmas will be used to test *in vitro* cross-reactivity of these different compounds. HIT diagnosis was confirmed in patients with both concordant PAT and ELISA positive tests.

To determine HIT cross-reactivity, using an optical aggregometer (Regulest, Nancy, France) at 37°C and 1100 rpm, we will perform PAT assay. Briefly, in the aggregometer cuvette, glass tube containing a magnetic bar, we will add 100 µl of HIT positive plasma to 170 µl of control PRP (final platelet concentration 300 G/l). After addition of various compounds (30 µl) (UFH, oligosaccharides, Pentasaccharide) at 3 different dosages (0.5, 1 and 100 anti-Xa IU/ml) or saline (negative control), aggregation profile will be studied during 15 min. If aggregation occurs, preincubation of test-platelets with a monoclonal antibody (IV-3, Medarex, USA), abolishing this platelet response, will confirm the implication of Fc membrane receptor CD32 in this positive reaction and prove its immunological origin. The PRP of the donor will be also tested to eliminate any unspecific platelet response.

PAT will be considered as positive if :

1/ we observe an increase of light transmission over 20% in the presence of studied compound as compared to saline.

2/ this aggregation is inhibited by a very high concentration of the same compound and/or IV-3 pre-incubation.

#### IV. RESULTS

We have studied thirteen various oligosaccharides provided by Aventis Pharma. We have compared their ability to provoke citrated platelet rich plasma aggregation in presence of HIT plasma to UFH (unfractionated heparin, Choay<sup>®</sup>), PS (pentasaccharide, Arixtra<sup>®</sup>), low molecular weight heparin (enoxaparin, Lovenox<sup>®</sup>) and SD (sodium danaparoid, Organon<sup>®</sup>).

#### Species Nomenclature

	<b>DIA2844</b> <b>(&lt; 7% 1, 6 Anhydro)</b>	<b>WSD3093</b> <b>(15-25% 1, 6 Anhydro)</b>
<b>&lt; HEXADECASACCHARIDE</b>	<b>C<sub>2</sub></b>	<b>C<sub>8</sub></b>
<b>≥ HEXADECASACCHARIDE</b>	<b>C<sub>3</sub></b>	<b>C<sub>9</sub></b>
<b>HEXASACCHARIDE</b>	<b>C<sub>4</sub></b>	<b>C<sub>10</sub></b>
<b>OCTASACCHARIDE</b>	<b>C<sub>5</sub></b>	<b>C<sub>11</sub></b>
<b>DECASACCHARIDE</b>	<b>C<sub>6</sub></b>	<b>C<sub>12</sub></b>
<b>DODECASACCHARIDE</b>	<b>C<sub>7</sub></b>	<b>C<sub>13</sub></b>

**C<sub>1</sub> = ENOXAPARIN POLYSACCHARIDES 1/30/04**

**Table 2. In vitro cross-reactivity in presence of various HIT plasmas (N=50)**

SPECIES	% Cross-Reactivity
UFH	100
PENTASACCHARIDE	0
ENOXAPARIN	72
SODIUM DANAPAROID	7
C1	69
C2	64
C8	67
C3	68
C9	65
C4	0
C10	0.5
C5	70
C11	68
C6	70
C12	65
C7	70
C13	72

## V. DISCUSSION AND CONCLUSION

All oligosaccharides but C4 and C10 showed an *in vitro* immune cross reactivity similar to enoxaparin. Both are **Hexasaccharides** and under the threshold size able to induce an immune response as described by Amiral (5). Immunoreactive complexes between PF4 and heparin are formed only under certain conditions. It depends on oligosaccharide composition, polysaccharide length and grade of sulfation (5). It was shown that formation of PF4-heparin complexes requires a heparin molecule with at least 12 oligosaccharide units and a high sulfation grade (more than 3 per disaccharide) (6).

Pentasaccharide also did not induce a platelet response in presence of neither HIT plasma as we have previously shown (4).

Using a standardized method of platelet aggregation test classically performed for HIT biological diagnosis, we demonstrated that there is no significant difference between WSD and DIA oligosaccharide structure regarding their ability to induce an immune cross reactivity with HIT antibodies. Only oligosaccharide below hexasaccharide size seem unable to induce an immune response.

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