Carbohydrates

— Synthetic Methods and Applications in Medicinal Chemistry

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20. Chemical and Enzymatic Methods for the Depolymerization and Modification of Heparin

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20.1 Introduction

This chapter examines heparin derivatives with new or improved properties prepared by chemical and enzymatic methods. Heparin was discovered by Jay McLean working with William Howell seventy-five years ago and first used clinically by Clarence Crafoord over a half-century ago. Despite its long history, the heparin routinely used today differs little from the drug recognized by Howell as showing great promise in the treatment of coagulation disorders. The decade of the eighties began with the discovery of the antithrombin III (ATIII) binding site, a specific pentasaccharide sequence with heparin capable of binding ATIII and responsible for much of heparin's anticoagulant activity. In the last ten years, the biosynthesis of heparin has been elucidated as well as many details of its intricate structure. It was during this period that synthetic heparin oligosaccharides were first prepared, and many new biological activities were discovered and clinical studies of low molecular weight (LMW) heparins were performed.

These recent advances in the basic understanding of the chemistry and biology of heparin combined with the successful clinical application of LMW heparins suggest that it is important to re-examine the methods, developed in the past, by which heparin derivatives can be prepared. These include both enzymatic and chemical methods aimed at improving the chemical and biological properties of heparin by decreasing its molecular weight or otherwise modifying its chemical structure. The goal of these methods is the preparation of heparin derivatives that are structurally more well-defined, have specific activities, improved bioavailability (in particular increased subcutaneous absorption) and pharmacokinetics, and increased potency with decreased side effects (a higher therapeutic index).

20.2 Heparin: Structure and Activity

Heparin is biosynthesized as a proteoglycan composed of a central core protein from which approximately ten linear, acidic polysaccharide glycosaminoglycan (GAG) chains extend. The biosynthesis of heparin begins in the rough endoplasmic reticulum with the synthesis of core protein having a large number of serine-glycine repeats. The linkage region is composed of neutral sugars attached to serine residues from which GAG chains extend. A repeating polymer of 1→4 linked glucuronic acid and N-acetylgalactosamine is assembled on this
linkage region. This polymer is then N-deacetylated, sequentially N- and O-sulfated and the glucuronic acid is epimerized to iduronic acid. The structural variability in the heparin polymer is primarily the result of the incomplete nature of these post polymerization modifications. Processing by proteases and endo-β-glucuronidases during storage and release of heparin from the mast cell granules results in a polydisperse mixture of glycosaminoglycan chains having a greatly reduced molecular weight and very similar in structure to the commercial drug form.  

Heparin glycosaminoglycan (henceforth referred to simply as heparin) has a molecular weight range of 5,000–40,000 with an average molecular weight of 10,000–15,000 and is composed of disaccharide units shown in Fig. 20.1. These disaccharide units are not arranged in a random sequence; instead their sequence is dictated by the specificity of the various enzymes involved in the biosynthetic pathway. One unique but rare sequence that occurs in heparin is the ATIII binding site (Fig. 20.1).  

Heparin’s major activity is as an anticoagulant. This anticoagulant effect prevents clot formation during extracorporeal therapy, such as kidney dialysis, or following surgery. However, excessive anticoagulation can lead to hemorrhage and sometimes death. Heparin acts primarily through its binding to ATIII. Heparin-bound ATIII undergoes a conformational change, observed by an enhancement in its fluorescence spectrum. This conformational change increases the ability of ATIII to inhibit thrombin, a serine protease responsible for conversion of soluble fibrinogen to an insoluble fibrin clot. Heparin also has activity towards other serine proteases, platelets and on the endothelium, which in combination represent its antithrombotic effect. A reduction in heparin’s molecular weight reduces the anticoagulant effect to a greater degree in comparison to the antithrombotic effect, and provides a rationale for the use of LMW heparins. These LMW heparins are claimed to have reduced hemorrhagic side effects (a higher safety/efficacy ratio) in addition to more predictable pharmacological action, sustained antithrombotic activity and improved bioavailability.  

Heparin has a number of other biological activities associated with its ability to bind, through ionic interaction, to a variety of important proteins and thus may be better termed

\[ \text{ATIII-BINDING SITE} \]

\[ \text{Fig 20.1: The major disaccharide unit in heparin is observed variation in disaccharide structure (a) and the structure of heparin’s ATIII binding site (b). Where } X = \text{SO}_3 \text{ or } H \text{ and } Y = \text{COCH}_3 \text{ or } \text{SO}_2. \]
a polyanionic drug than simply an anticoagulant. Heparin's anti-atherosclerotic activity is related to its capacity to stimulate the release of lipoprotein lipase from the endothelium and to activate it to decrease the concentration of atherogenic lipoproteins. Heparin also inhibits the smooth muscle proliferation that follows damage to the endothelium, thus decreasing atherogenesis. Although the mechanism of this antiproliferative effect is still not well understood, it may involve heparin's interaction with growth factors. Heparin inhibits complement activation and may play a role in the normal regulation of the immune response. Angiogenesis or the formation of new blood vessels, is regulated by heparin. This activity may give heparin a role in processes where new vessel growth occurs, including wound healing, tumor growth, ovulation and fetal development. Heparin is an antiviral agent demonstrating anti-HIV activity. Heparin also binds many other proteins, including enzymes, activating, inhibiting or protecting their activity.

20.3 LMW Heparins

The most important of the heparin derivatives from the standpoint of clinical use as antithrombotic agents are the LMW heparins. LMW heparins are modified heparin fractions consisting of GAG chains of molecular weights ranging from 2,000 to 8,000 (average of 5,000). LMW heparin can be prepared by fractionation of commercial heparin or by its controlled chemical or enzymatic depolymerization. Although the major application of LMW heparin has been as antithrombotic agents, other uses can be envisaged where the other biological activities of heparin are exploited. These alternative applications may also include smaller heparin oligosaccharides. While LMW heparin is a polydisperse mixture, small oligosaccharides can be prepared that are pure, discrete chemical entities of defined structure.

It is important to note that LMW heparins are mixtures and each possesses unique biological properties depending on the content and structure of its components. Although a LMW heparin is primarily described by its molecular weight and polydispersity, the distribution and frequency of individual chains found in the mixture and their sequence are also important. The content and structure of a LMW heparin are related to the species and tissue source of the parent heparin, the method and conditions of its depolymerization, and the fractionation methods used in processing the LMW heparin. A variety of methods have been used to prepare LMW heparin and heparin oligosaccharides but only a few of these are used preparing commercial products.

20.3.1 Fractionation

Commercial glycosaminoglycan heparin is polydisperse and contains <15 wt% chains having MW≤5,000. The content of low molecular weight chains in commercial heparin can be enriched by solvent precipitation or by gel permeation chromatography. However, because of the small percentage of LMW chains present in commercial heparin, the yields are low and the resolution of these fractionation methods is poor. LMW heparin CY216 (Sanofi, France) was at one time prepared by ethanol precipitation of commercial heparin but is now prepared by depolymerization methods as a result of the problems associated with heparin fractionation.
20.3.2 Acid Hydrolysis

Heparin can be depolymerized by hydrolysis using hydrochloric acid (Fig. 20.2). Using 0.2M hydrochloric acid for 24h at 100°C results in the formation of desulfated, deacetylated oligomers, ranging from disaccharide to pentasaccharides. Acid hydrolysis proceeds preferentially by N-desulfation followed by hydrolysis of glycosidic linkages to iduronic acid residues, the hydrolysis of glycosidic linkages to other sugar residues, and ultimately the hydrolysis of O-sulfate and N-acetyl groups. The lability of the glycosidic linkages in heparin can be increased by carbodiimide activation of iduronic acid 2-sulfate and its reduction to iditol 2-sulfate using sodium borohydride. Hydrolysis of this reduced heparin takes place with 0.1M hydrochloric acid at 25°C and yields primarily disaccharide products that retain some O-sulfate and N-acetyl groups. These products, however, contain reduced uronic acid residues.

The loss of labile N-sulfate groups during acid hydrolysis can be circumvented by hydrolyzing glycosidic linkages in the presence of a sulfating agent. Heparin is first dried extensively, then treated with 95% sulfuric acid and 5% chlorosulfonic acid at -4°C, poured on cold diethyl ether to precipitate the heparin, and neutralized. This results in the formation of

![Chemical structures](image-url)

**Fig. 20.2** Products of acid hydrolysis of heparin using strong acid (a), by reduction of carboxyl groups followed by mild acid treatment (b), and using chlorosulfonic acid and sulfonic acid (c); where n = 0, 1, 2…; Z = SO₂ or H, Y = SO₂ or COCH, and Z = H or COCH.
a supersulfated LMW heparin. The additional sulfate groups resulting from the reaction of heparin with chlorosulfonic acid are found at both the 3-positions of iduronic acid 2-sulfate and glucosamine 2,6-disulfate. Supersulfated LMW heparin retains substantial pharmacological activity.

20.3.3 Chemical β-Elimination

The uronic acid residues in heparin contain an acidic proton at C-5, α to the carboxylate group. The acidic proton at C-5 is β to the C-4 glycosidic linkage. In iduronic acid (C6 chair form) this acidic proton at C-5 is in an anti-orientation to the C-4 glycosidic linkage. Thus, under strongly basic conditions facile β-elimination takes place with cleavage of this glycosidic linkage resulting in the formation of a Δ4,5 unsaturated uronic acid (Fig. 20.3). The negative charge present on the ionized carboxylate group decreases the rate of base catalyzed β-elimination since it requires the formation of a dianion intermediate. Esterification of iduronic acid removes the negatively charged carboxylate group, increasing the rate of β-elimination. Thus esterification prior to β-elimination permits the use of mildly basic conditions that do not promote side reactions that damage other sensitive functional groups in heparin. One example of such a side reaction is the base-catalyzed intramolecular displacement of 2-sulfate by the 3-hydroxyl group in iduronic acid 2-sulfate, forming a 2,3-epoxyuronic acid.

Benzyl esters are often chosen as protecting groups because of their ease of removal. The benzyl ester of heparin is prepared by treating the benzethonium salt of heparin (soluble in

![Diagram](attachment:diagram.png)

Fig. 20.3 Chemical β-eliminative cleavage of heparin by esterification (a) and treatment with base (b) where \( n = 0, 1, 2 \ldots \), \( R = \text{benzyl or methyl} \), \( X = \text{SO} \), or H, and \( Y = \text{SO} \), or \( \text{COCH}_3 \).
Fig. 20.4  Mechanism for the deaminative cleavage of heparin where X=SO$_3^-$ or H.
20.3.4 Deaminative Cleavage

The glucosamine residue in heparin is susceptible to nitrosation forming an unstable N-nitrosog derivative that on rearrangement can lose nitrogen and forms a carbocation at the C-2 position (Fig. 20.4). Nucleophilic attack by the ring oxygen on this center results in ring contraction and cleavage of the adjacent glycosidic linkage. This reaction has been used to study heparin's structure and also to prepare LMW heparins having biological activity.

Treatment of heparin with nitrous acid at pH 1.5 (without prior N-desulfation) cleaves glycosidic linkages to glucosamine and all N-sulfated glucosamine residues. At pH 4.0, nitrous acid treatment only cleaves linkages to glucosamine or glucosamine 6-sulfate. These residues are often enriched prior to deaminative cleavage by N-desulfation. The uronic acid residue, glycosidically linked to the C-4 of the glucosamine undergoing ring contraction, is believed to be unaltered.

The 2,5-anhydro-β-mannose residue formed at the reducing end of the oligosaccharide is usually stabilized by reduction with sodium borohydride to anhydromannitol. The 2,5-anhydro-β-mannose residue in an oligosaccharide or in LMW heparin can also be used to link it to an amino-containing polymer surface through reductive amination. Alternatively, the aldehyde group, in the 2,5-anhydro-β-mannose, can be stabilized by oxidation to a carboxylate group with basic potassium permanganate.

Nitrous acid treatment for the partial depolymerization of heparin has been used to prepare several commercial LMW heparins including Fraxiparin and CY 222 (Sanofi, France), Fragmin (KabiVitrum, Sweden), and standard LMW-heparin (NIBSC, England).

20.3.5 Periodate/Oxidative/Free Radical Cleavage

A variety of oxidative/peroxidative methods have been used to depolymerize heparin (Fig. 20.5). Smith degradation is a classical method that involves periodate oxidation followed by borohydride reduction and mild acid hydrolysis. The unsulfated glucuronic acid and iduronic acid residues in heparin having vicinal diol functionality are susceptible to periodate oxidation. The small number of unsulfated uronic acid residues reduces heparin's susceptibility towards this method of depolymerization.

A second process relies on hydrogen peroxide at low pH to oxidatively depolymerize heparin. Heparin (sodium salt) is first converted to heparinic acid using the H+ form of a cation exchange resin, then treated with hydrogen peroxide in an autoclave. After adjusting the pH to 7, the LMW heparinamide (N-desulfated LMW heparin) is then resulfated using pyridine/sulfur trioxide in pyridine. RD-heparin (Hepar, US) is currently in clinical trials in the United States. This LMW heparin is prepared by a modification of this process; run under milder conditions, in which N-desulfation is eliminated and resulfation is not required.

A free radical process involving hydrogen peroxide at neutral pH has also been used to depolymerize heparin. Heparin is treated with ascorbate, Cu2+, and hydrogen peroxide at pH 26
Fig. 20.5 Products of periodate/oxidative and free radical cleavage of a particular sequence within heparin by Smith degradation (a), by hydrogen peroxide at low pH (b), by modified hydrogen peroxide cleavage under mild conditions (c) by copper, ascorbate and hydrogen peroxide at neutral pH (d) and by Fe$^{3+}$ and O$_2$ or hypochlorous acid (e).
[at pH 3.5 no reaction takes place] to generate hydroxyl radicals, which ultimately depolymerize heparin. This process results in no loss of sulfate groups, thus the LMW heparin formed has a sulfate/carboxylate ratio identical to the parent heparin. The formation of chains (in the above process) having both even and odd numbers of saccharide units suggests that cleavage occurs at both hexosamine and uronic acid residues. Although the mechanism for this reaction is not completely understood, the degree of depolymerization can be influenced by controlling a variety of parameters including reaction time, temperature and pH. The commercial LMW heparin Flouxim (Opocrin, Italy) is made from bovine intestinal mucosal heparin using this process.

A second free radical-based depolymerization, called oxidative reductive depolymerization (ORD), using oxygen at neutral pH to depolymerize heparin has recently been reported. Heparin in 200 mM phosphate (pH 7.2) is incubated at 50°C under an oxygen atmosphere for 20 h in the presence of Fe²⁺. Under these conditions, Fe²⁺ is oxidized to Fe³⁺ with the formation of hydroxyl radicals. These oxygen-derived free radicals are believed to be capable of destroying all types of monosaccharide residues in heparin, with the exception of iduronic acid 2-sulfate. Thus, when this reaction is carried out under controlled conditions, the average molecular weight of heparin can be reduced from 12,000 to 3,000. Little or no loss of anticoagulant activity is observed and significant anticoagulant activity remains. Depolymerization is believed to take place through the destruction of unit monosaccharides, followed by secondary hydrolysis similar to the ORD reaction of hyaluronic acid.

A similar method for preparing LMW heparin, not requiring the use of metals, relies on hypochlorous acid to generate oxygen radicals. Heparin dissolved in citrate/phosphate buffer at pH 6.2 is treated with hypochlorous acid at 80°C to reduce heparin's molecular weight by 50% in less than 1 h. While the anticoagulant activity is also reduced by 50%, the goal is to improve both bioavailability and antithrombotic activity.

### 20.3.6 Enzymatic β-Elimination

Heparin can be depolymerized enzymatically under very mild conditions using polysaccharide lyases (Fig. 20.6). Polysaccharide lyases (E.C. 4.2.2.1) act eliminatively to break the glycosidic linkages between hexosamine and uronic acid giving a new hexosamine reducing end and an unusual, unsaturated uronic acid at the non-reducing end. There are three commercially available polysaccharide lyases that are capable of acting on linkages within heparin: heparin lyase (EC 4.2.2.7), heparitinase (EC 4.2.2.8) and heparin lyase II (no EC number). Although the specificity of these enzymes is not completely understood, studies on heparin, heparan sulfate and structurally characterized heparin oligosaccharides have led to an understanding of the linkages susceptible to enzymatic cleavage (Fig. 20.6). These enzymes are prepared fermentatively from Flavobacterium heparinum, an organism that also produces exoglucuronidases, sulfotransferases and sulfatidases. The lyase enzymes act under mild conditions at temperatures between 20°C and 40°C at pH 6–8 to depolymerize heparin. The particular enzyme preparation used to depolymerize heparin must be sufficiently purified so that it does not contain other enzymatic activities.

Purified heparin lyase has been used to prepare LMW heparins with retention of anticoagulant activity. A commercial LMW heparin, Logiparin (Novo, Denmark), is also prepared using this method. Although a mild method of depolymerization, a major disadvantage of this method is the unusual unsaturated uronic acid residue found in the nonreducing end of the
Fig. 2.16 Products of enzymatic β-eliminative cleavage of heparin using heparinase (EC 4.2.2.7), also called heparin lyase I (a), heparin lyase II (b), and heparinase (EC 4.2.2.8), also called heparin lyase III (c), where \( n = 0, 1, 2 \ldots \), \( X = SO_3 \) or H, and \( Y = SO_3 \) or COCH.\_
20.4 Chemically Modified Heparins

20.4.1 Reactions at Carboxylate Groups

There are four different types of uronic acids residues found in heparin: (i) iduronic acid, (ii) iduronate 2-sulfate, (iii) glucuronic acid, and (iv) glucuronic acid 2-sulfate. The pKa of all these uronic acid carboxylate groups, except for glucuronic acid 2-sulfate, has been determined by 1H-NMR. The differences in their acidity make selective derivatization possible. Selective formation of methyl ester of iduronic acid or iduronate 2-sulfate has been reported. Esterification of the benzethonium salt of heparin with benzyl chloride and the sodium salt of heparin with diazomethane has been reported. Alternatively the carboxyl group can be activated with carbodiimide and imidazol with an amine, such as glycine methylester, or reduced to an alcohol using sodium borohydride.

20.4.2 Reactions at Hydroxyl Groups

Heparin is highly sulfated and contains 2.5–3.0 sulfate groups per disaccharide repeating unit. The glucosamine residue is typically N-sulfated and O-sulfate groups are often present at the 6-position and occasionally at the 3-position. The 2-position of iduronic acid is usually sulfated. The C-2 and C-3 hydroxyl groups of glucuronic acid and iduronic acid are most available for derivatization. The C-3 of iduronic acid 2-sulfate and the C-3 of glucosamine residues are also available for derivatization but are generally less reactive. Esterification of the cetyltrimethylammonium salt of heparin can be carried out in organic solvents using simple acid chlorides. Alternatively the sodium salt of heparin is soluble in formamide and has been esterified using acetic anhydride as well as acetic anhydride.

Heparin pyridinium salt in dimethyl sulfoxide can be chemically O-desulfated with some selectivity. For example, the 2-O-sulfate groups are more labile than the 6-O-sulfate groups of heparin containing N-acetylated glucosamine residues but the opposite selectivity is observed for heparin containing unsubstituted glucosamine residues. Alternatively, specific O-sulfoesters have been prepared that act on heparin-derived oligosaccharides. These include a 6-O-sulfoesterase (EC 3.1.6.11) that acts on N-acetylated (or N-sulfated) glucosamine-6-sulfate and a 3-O-sulfoesterase (EC 3.1.6.-) that acts on N-acetylated (or N-sulfated) glucosamine-3-sulfate residues.

Heparin can also be selectively O-sulfated. Whole intestinal heparin, having a low degree of O-sulfation, is selectively 6-O-sulfated as the tributylammonium salt in dimethylformamide. This modification significantly increases anticoagulant activity. Similar increases in anticoagulant activity have been reported when the pyridinium salt of heparan sulfate (also having a low level of both N- and O-sulfation) is chemically sulfted.

Epoxidation of heparin can be accomplished by lyophilizing sodium heparin at pH 11.5
Fig. 20.7  Epoxidation of heparin by iyoophilization of the sodium salt under alkaline conditions. The epoxide can be opened by attack of a nucleophile (Z) at either the 3-position (a) or the 2-position of the epoxysudonic acid, where \( X = \text{SO}_3^- \) or \( H \), and \( Y = \text{SO}_3^- \) or \( \text{COCH}_3 \).
20.4.3 Reactions at Amino Groups

*N*-desulfation is easily accomplished by heating the pyridinium salt of heparin in dimethyl sulfoxide. Enzymatic *N*-desulfation of heparin-derived oligosaccharides is also possible by using 2-deoxy-2-sulfamido-6-glucose sulfamidase (EC 3.10.1.1). Although most amino groups within heparin are sulfated, porcine mucosal heparin contains a significant number of acetylated amino groups. N-acetyl groups can be removed by treatment with sodium hydroxide, but even under the mildest conditions that release N-acetate, significant structural damage occurs within the polymer possibly through \( \beta \)-elimination. This structural damage caused by the use of alkaline conditions is demonstrated by the inability of heparin lyase to utilize base-treated heparin as a substrate even after *N*-reacetylation. A screening of ten different commercially available proteases and peptidases at very high concentrations showed that none could catalyze the *N*-deacetylation of heparin. Treatment of heparin with hydrazine followed by workup of the product with iodide acid to destroy the uronic acid hydrazides yields *N*-deacetylated heparin. This product was heparinase-sensitive following *N*-reacetylation. Free amino groups are readily derivitized, for example, by *N*-acylation with acetic anhydride aqueous sodium carbonate or *N*-sulfated using triethylamine-sulfur trioxide.

Recently, Uchiyama and Nagaosawa described a new reaction in which the pyridinium salt of dry porcine heparin is heated in vacuo to effect the transfer of *N*-sulfate groups preferentially to the 3-hydroxyl groups of the polysaccharide. Subsequent *N*-resulfation results in an oversulfated heparin with enhanced anticoagulant activities.

20.4.4 Reactions at the Reducing End

Reduction of heparin's reducing end with sodium borohydride provides a useful method of introducing radiolabel at a specific site. This reduction is a very inefficient reaction requiring a large excess of reducing agent. Studies using structurally defined heparin oligosaccharides having *N*-sulfated glucosamine 6-sulfate at their at their reducing end, however, demonstrated that this reaction could be carried to completion affording a high yield of the expected products. Heparin and heparin oligosaccharides can also be fluorescently tagged at the reducing end through reductive amination (Fig. 20.8). Heparin or heparin oligosaccharide is first incubated with 7-amino-1,3-naphthalene disulfonic acid monopotassium salt to form the Schiff base, which is then reduced using sodium cyanoborohydride. The expected products can be isolated in good yields using this method.
20.5 Immobilization of Heparin

Heparin has been covalently immobilized to polymeric matrices using a variety of chemical linkages such as (i) an amide through its carboxyl groups by carbodiimide activation; (ii) an amide to carboxylated supports or to cyanogen bromide activated supports through free amino groups prepared by N-desulfation or N-deacetylation or by attaching an amino containing coupling arm; (iii) an ether through hydroxyl groups reacting with epoxidated supports; (iv) linking heparin at its reducing end by reductive amination or through an anhydromannosamine residue produced using nitrous acid; and (v) through direct polymerization of a vinyl or acrylated heparin derivative to form an insoluble polymer.

Although all of these methods are effective at immobilizing heparin, matrices prepared by each method have different physical, chemical and biological properties. There are a few important aspects with respect to an immobilized heparin that need to be considered, e.g. (i) chemical and enzymatic stability (particularly important for biomedical device applications); (ii) the quantity of the heparin that can be coupled; (iii) the maintenance of biological activity; (iv) the orientation of the coupled chains; (v) density and uniformity of polymer coverage; (vi) the physical, chemical and biological properties of the underlying polymer. Additional studies relying on structurally defined heparin oligosaccharides will be required to address these issues.

20.6 Future Prospects and Conclusions

Heparin has been in continuous clinical use for over half a century. Despite the hemorrhage complications associated with its use, no replacement for this very potent and effective drug is on the horizon. The discovery of many new biological activities of heparin has been
reported in the past decade spurring additional research on heparin. Immobilized heparins are also becoming more important with the increased demand for biomedical devices and artificial organs that require heparin or heparinized biomaterials. All these factors suggest that heparin will be widely used well into the twenty-first century.

The chemical and enzymatic methods described in this chapter have been developed primarily to determine heparin's structure since its discovery in 1916. These methods now need to be re-examined with the goal of preparing improved LMW heparins and modified heparins. The use of heparin oligosaccharides, with defined chemical structure, as model compounds is essential to further develop heparin chemistry.

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62. Unpublished data from our laboratory.