Chemistry and Biology of Heparin Mimetics that Bind to Fibroblast Growth Factors

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Abstract: We aim from this review to stimulate further research in this area by providing a description of the different types of inhibitors containing heparin mimetic molecules that have recently been reported and data on their biological activity. Molecules that mimic heparin and bind to heparin-binding growth factors are important building blocks for synthetic biomaterials. Different types of synthetic mimics of the biological properties of heparin have been prepared including high molecular weight compounds or small molecule mimics. Peptide-based mimics of heparin functionality are limited and because of their low degree of sulfation, they are natural targets as heparin mimics. Aromatic sulfonamide derivatives exhibit a range of bioactivities and a novel angiogenesis inhibitor (E 7820) is used as a TF model for screening assay. The anticoagulant activity of the known heparin pentasaccharide sequence prompted synthetic efforts aimed at the procurement of this structure as well as a host of related sequences. Chemical modification of the natural or synthetic heparin increased factor activation of AT III Xa affinity. A variety of non-peptide non-saccharides inhibitors as anti-angiogenesis therapies directed against the VEGFR kinase are a promising and well-validated therapeutic approach under active evaluation of their safety and efficacy in multiple clinical trials. These low molecular weight modulators could be useful tools for biologists and may have potential as drugs or as leads for drug development.

Key Words: FGF family, heparin, angiogenesis, tumor, peptides, glycosides, polymers, inhibitors.

1. INTRODUCTION

The fibroblast growth factors (FGF) are a large family of polypeptides molecules with many diverse biological activities both *in vitro* and *in vivo*, in embryonic development and in adult tissues. The FGF family consists of at least 19 members that are characterized by structural similarity in amino acid ranging from 40 to 70% sequence identity [1]. On the basis of the structural similarity and biological activity between FGFs, a number of related sub-families have been identified within the whole family. For instance, FGF that has the ability to bind with heparin designated as FGF-1 and FGF-2 or heparin-binding growth factors HBGF-1 and HBGF-2 or acidic and basic fiber growth factors aFGF and bFGF respectively. This subfamily is identified as a 155 amino acid residues sequence about 55% homologous. FGF-subfamily members (FGF-3 to FGF-6) derived from oncogenes and identified in tumor cell lines as, respectively, a 239 amino acid sequence with about 90% homologous to its murine counterpart [2], a 206 amino acid sequence with about 45% homology to the sequence of FGF-2 [3-6], a 267-268 residues with 44% homology to FGF-2 [7], and a 198 residues almost identical to FGF-4 [8,9]. The remaining FGF subfamilies are named FGF-7 to FGF-19 and there are a number of reviews describing these polypeptides [10-14].

aFGF (or HBGF-1) and bFGF (or HBGF-2) are the most widely studied fibroblast growth factors [15-18]. Both are two related peptides that were identified and purified from different tissues [19-21]. The structure of these FGF is consisting of 12 anti-parallel β-strands arranged in a pattern with approximate *C3* internal symmetry. Crystallographic studies, in combination with experiments of site directed mutagenesis [22] led to the identification of several regions participating in cell attachment (receptor binding site) and heparin binding (heparin binding site). In particular, a highly positively charged region consisting of a β-turn, a short β-strand, and a loop, containing five basic residues (Lys119, Arg120, Lys 125, Lys129, and Lys135 in FGF-2), was suggested as the binding site for heparin and other sulfated substrates. Binding of FGFs to heparin and other glycosaminoglycans protects them from degradation and can retain FGFs in the extracellular matrix as a reservoir. These growth factors bind to cell surface heparan sulfate proteoglycans, but it is not completely clear whether these are functional receptors.

Heparin is one class of glycosaminoglycans (GAGs) [23], a major class of extracellular complex polysaccharides that represents the primary constituents of every eukaryotic cell surface and the extracellular environment. Classes of GAGs include (Fig. **1**): a) heparin (HP) and heparan sulfate (HS), **1-4** contain repeats of the disaccharide [IdoAα(1→4) GlcNAc] interspersed with its epimer [GlcAβ(1→4)GlcNAc], all connected by α(1→4) linkages. Heparin differs from heparan sulfate in that it is more highly sulfated and contains more of the isomeric variant IdoA; b) chondroitin sulfate (CS) **5-7** is a repeat of the disaccharide [GlcAβ(1→3)GalNAc] connected by β(1→4) linkages; c) dermatan sulfate (DS) **8-11** is similar to chondroitin sulfate but also contains some isomerically different disaccharides in the form of [IdoAα(1→3)GalNAc]; d) keratan sulfate has a repeating sequence of the disaccharide [Galβ(1→4)GlcNAc] connected by β(1→3) linkages; e) hyaluronan **12-13** is an invariant GAG made up of repeats of the disaccharide [GlcAβ(1→3) GlcNAc] connected by β(1→4) linkages. With the exception

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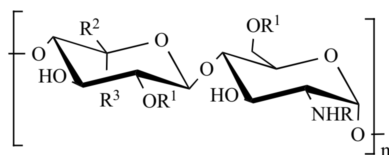
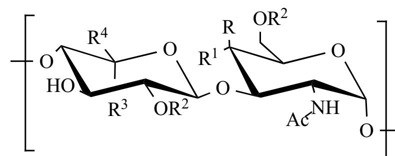


Heparin (HP)
Heparan sulfate (HS)

1 R = R^1 = H or SO_3^-, R^2 = H, R^3 = COO^-
2 R = R^1 = H or SO_3^-, R^3 = H, R^2 = COO^-
3 R = H or Ac or SO_3^-, R^1 = H or SO_3^-, R^2 = H, R^3 = COO^-
4 R = H or Ac or SO_3^-, R^1 = H or SO_3^-, R^3 = H, R^2 = COO^-



Chondroitine sulfate (CS)
5 R = OH, OSO_3^-, R^1 = H,
6 R^2 = H or SO_3^-
7 R^3 = H, R^4 = COO^-

Hyaluronic acid (HA)
12 R = R^2 = R^3 = H,
13 R^2 = OH, R^4 = COO^-

Dermatan sulfate (DS)
8 R = OH, OSO_3^-, R^1 = H,
9 R^2 = H or SO_3^-
10 R^3 = H, R^4 = COO^-
11 R^4 = H, R^3 = COO^-

Fig. (1).

β(1→4) linkages. With the exception of hyaluronic acid and heparin, GAGs are usually covalently attached to a protein core, forming an overall structure referred to as proteoglycan (PG).

The interaction between the FGF and their tyrosine kinase receptors FGFR subtypes depends on the sequence of interacting HS chains. Structural information available for FGF is known to bind with the repeating disaccharide units of HS. It binds to HS chains that are as small have a pentasaccharide, but it requires *N*-sulfation of each of the glucosamine units and one iduronate-2-sulfate unit [24]. The specific amino acid residues on FGF that are important for the binding of the protein to the GAG have been also determined and, as with most heparin-binding proteins, include a large percentage of basic amino acids [25]. The other well-studied example of protein-GAG interactions involves the binding of antithrombin to heparin/HS, which results in the inactivation of the coagulation cascade. In contrast to FGF, antithrombin is known to bind a relatively rare modification site on HS that consists of a pentasaccharide containing a disaccharide unit of 3-*O*-sulfated glucosamine with an uronic acid, attache d to the non-reducing end of a fully modified disaccharide [26]. Additionally, whereas the structure of FGF remains rela-tively unchanged upon complexation with heparin saccharides, antithrombin undergoes a conformational change, exposing a loop that makes it a potent inhibitor of thrombin [27].

Despite its widespread use, heparin is heterogeneous, difficult to modify, and limited in clinical use as a drug due to the side effects that result from undesirable interactions. It would thus be interesting to search for compounds that could specifically target heparin-binding proteins. The article of Jirmanova *et al.* [28], which revealed that oligosaccharides containing Lewis X structural motifs have effects on proliferation of embryonic stem cells possibly through the modulation of FGF-2 (bFGF), further suggesting a potential role for smaller molecules in modulating FGF activity. In other words, FGF can recognize structural features of smaller non-sulfated oligosaccharide components of heparin/heparan sulfate and provide a platform for the development of small molecule promoters or inhibitors of FGF function [29].

The goal of this review is to stimulate further research in this area by providing a description of the different types of inhibitors containing heparin mimetic molecules that have recently been reported, and data on their biological activity whenever such data are available. These low molecular weight modulators could be useful tools for biologists and may have potential as drugs or as leads for drug development.

2. HEPARIN MIMETIC MOLECULES

Molecules that mimic the sulfated glycosaminoglycan heparin and bind to heparin-binding growth factors would serve as important building blocks for synthetic biomaterials [29]. At this point, one would ask is there an inherent structural limitation that precludes the interactions between proteins and the mimetic molecules? Indeed, on any given protein surface there is a multitude potential interaction sites that are as diverse as hydrophobic patches, hydrogen bond acceptors and donors, and hidden crevices or pockets that could open and engulf propitiously positioned substituents of the proper size. Combinatorial library studies for an array of protein interaction domains generally arrive at the same conclusion: even the highest affinity molecules identified from these libraries exhibit dissociation (or inhibitory) constants that lie at the AM/ nM border. The result is surprising given the fact that there are innumerable examples of active site-directed tight binding (<nM) low molecular weight compounds. What is the source of this apparent discrepancy? There are a number of possible explanations. Active sites are generally small, structurally well-defined pockets or crevices containing functional groups oriented in a precise fashion. A properly designed inhibitor contains its own functionality that is configurationally and conformationally complementary to those present in the active site. In addition, complex formation with an optimized inhibitor often results in the extrusion of water from the active site. The latter favors the formation of high affinity electrostatic and hydrogen bonding interactions that would be comparatively weak in the aqueous milieu. Finally, small molecules tend to possess relatively few degrees of structural freedom, thereby limiting the damaging effect of entropy on the dissociation constant.

Different types of synthetic mimics of the biological properties of heparin have been prepared including high molecular weight compounds such as sulfonated polymers, polyaromatic anionic compounds, polymers with amino acid side chains, polymers with mono- and disaccharide side chains or small molecule mimics include β-cyclodextrin tetradecasulfate, suramin and its analogs, and molecules based on derivatives of 2-(3-nitrobenzoyl)benzoic acid and acylamino acid amides. Among such compounds are also pentosan polysulfate [30], laminarin sulfate [31], sucrose octasulfate [32, 33], and aurin tricarboxylic acid (ATA) [34].

2.1. Heparin-Mimicking Peptides

Peptide-based mimics of heparin functionality have been limited. Compared to heparin and many mimics, the degree of sulfation in the peptides is low and thus they are natural targets as heparin mimics. Typically no more than four amino acids in the entire protein are sulfated. Peptides could be coupled with the relative ease to biomaterials and may exhibit lower toxicities compared to compounds such as suramin. Recent reports showed that in some cases sulfated proteins and GAGs bind to the same partners [35, 36]. These results suggest that synthetic peptides with only a few sulfates could potentially bind to the GAG partner proteins. Based on this concept, *Hubbell* and coworker [37] reported a rationally designed combinatorial approach to synthesize a 6600 members library of tetrapeptides by solid-phase Fmoc chemistry (Fig. **2**). Sulfated tyrosine Y-SO_3 **14**, aspartic acid (D) **15**, glutamic acid (E) **16**, and serine (S) **17** were included in the library. Since hydrophobic interactions may also be important, the amino acids alanine (A), valine (V), isoleucine (I), and phenylalanine (F) were also included, along with glycine (G). According to the fluorescent signal (Table **1**) that employed a 7-amino-4-methlcoumarin-modified $VEGF_{165}$, the sequence $SY(SO_3)DY(SO_3)$ was identified as the top binder and this peptide may be useful as alternatives to heparin where binding of VEGF is desired.

Ueki *et al.* [38] reported the solid-phase synthesis of oligotyrosine sulfates containing peptides and their biological activity. Synthesis of oligotyrosine sulfates was performed by oligomerization of tyrosine with simultaneous sulfation using $SO_3.Me_3N$ complex. From the mixture containing many products the salt of nonatyrosine-*N*- and O^{19}-decasulfate, NaO_3S-[Tyr(SO_3Na)]$_9$-ONa, was isolated and its

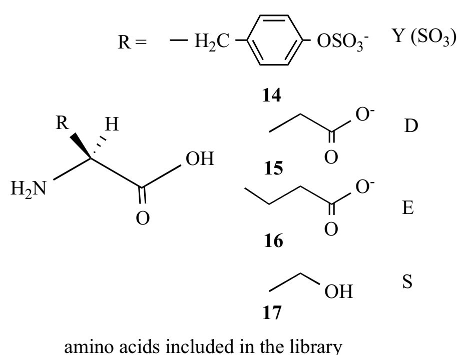


Fig. (2).

Table 1. Identification of Tetrapeptide VEGF165 Binders

Fluorescent signal[a]	Sequence
64	$SY(SO_3)DY(SO_3)$
61	$SY(SO_3)DY(SO_3)$
60	$AY(SO_3)DY(SO_3)$
52	$[S,G]Y(SO_3)Y(SO_3)Fb$
51	$SY(SO_3)AY(SO_3)$
46	$GY(SO_3)AY(SO_3)$
0	DIDF

a Fluorescent signal is $\sum Pix/r^{2.35}$.

b The first amino acid in this sequence is either S or G.

biological investigation revealed that it has the ability to inhibit human immunodeficiency virus infectivity. This result encouraged the same research lab [39] to synthesize a series of fully *N*- and *O*-sulfated homooligomers from octamer to nonadecamer of tyrosine, NaO_3S-[Tyr(SO_3Na)]$_n$-ONa (n = 8-19). The anti-HIV activity was found to be increased along with the increase of the chain length up to the dodecamer, then maintaining the same level to the length of the heptadecamer and then decrease.

Synthetic peptide, derived from a segment of the predicted amino acid sequence [40], C(cysteine)-R(arginine)-P(proline)-K(lysine)-A-K-A-K-A-K-A-K-D-Q(glutamine)-T (threonine)-K that binds to a HP sequence, was synthesized on a Vega 250 peptide synthesizer using fluorenylmethoxy-carbonyl (Fmoc) methodology [41]. Results revealed that the synthetic peptide was shown to compete with AT-III for binding to heparin and to neutralize the anticoagulant activity of heparin in blood plasma assays. Furthermore, the heparin fraction that binds to the synthetic peptide with high affinity exhibits an extremely high anticoagulant activity. Interestingly, despite the synthetic peptide shows no sequence similarity with AT-III, the two proteins recognize the same or similar structural motifs in heparin.

A straightforward automated method to create potent ligands for SH2 and SH3 domains, as well as powerful and highly selective inhibitors for protein kinases and phosphatases have recently been developed [42]. SH2 and SH3 domains are two types of many different structural motifs that have evolved to mediate protein–protein interactions [43,44]. These domains have one common functional feature, namely the ability to recognize and bind to amino acid sequences containing a phosphotyrosine moiety. They have been identified as potential therapeutic targets for the treatment of various cancers and autoimmune disorders, osteoporosis, anemia, and inflammatory diseases [45,46]. The library-based strategy was the preparation of reagents that selectively contend with a protein surface that is comparatively large, ill defined, and solvent exposed. As outlined in Scheme **1**, the initial step is the solid phase synthesis of a consensus sequence containing peptide. The peptide is prepared on a chemically altered Tenta Gel resin and the two are linked *via* a reducible disulfide bridge. The consensus peptide does

differ in one notable fashion from conventional peptides. 2,3-Diaminopropionic acid (''Dap'') is inserted within the consensus sequence at predetermined sites (vide infra). The amine side chain functional group serves as the attachment site for a wide variety of carboxylic acid derivatives, which vary in size, functionality, charge, polarity, and other features. As outlined in Scheme **1**, peptide **18** (Arg–Ala–Leu–Pro–Pro–Leu–Pro) was employed as the starting point for acquiring the targeted ligand. A series of modified Dap-containing libraries were prepared in a stepwise fashion. The K_D of the parent peptide domain complex is 15 ± 4 μM. N-terminal modification furnished **19**, which enjoys a 10-fold enhanced affinity relative to **18**. Analogous 10-fold improvements were obtained in each of the subsequent steps of the library scanning process. The final lead ligand, compound **21**, exhibits a K_D of 25 ± 0.05 nM, a 600-fold enhancement relative to the starting consensus peptide **18**.

In a similar manner, Inhibitors of protein kinase C (PKC) was designed by slightly modified the known consensus substrate sequence for PKC (-Arg–Arg–Lys–Gly–Ser–Hyd–Arg– (where Hyd = Phe/Leu/Ile/)) [47] to the closely analogous non-phosphorylatable peptide Ala-Arg-Arg-Gly-Ala–Leu–Arg–Gln–Ala, in which the Ser residue is replaced by Ala. Parent compound contains a simple acetylated Dap moiety exhibits a modest K_i value of 350 ± 80 nM. The 4-pyrrole phenylacyl Dap derivative is more than 600-times as potent (K_i = 550 ± 70 nM). Slight one N-terminal modification by changing the N-Ac to N-dichlorophenylacetyl group gave the lead derivative which exhibit a K_i value of 800 ± 25 pM. The overall enhancement from consensus sequence peptide 14 to lead inhibitor is nearly six orders of magnitude.

2.2. Suramin and Its Heterocyclic Analogues

Suramin **22**, (Fig. **3**), is a polysulfonated naphthylurea compound that has been widely used for the treatment of

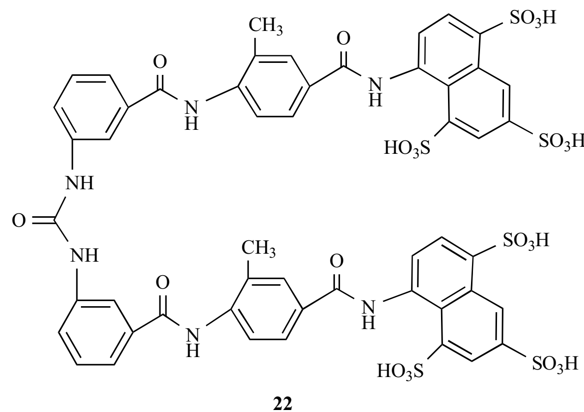
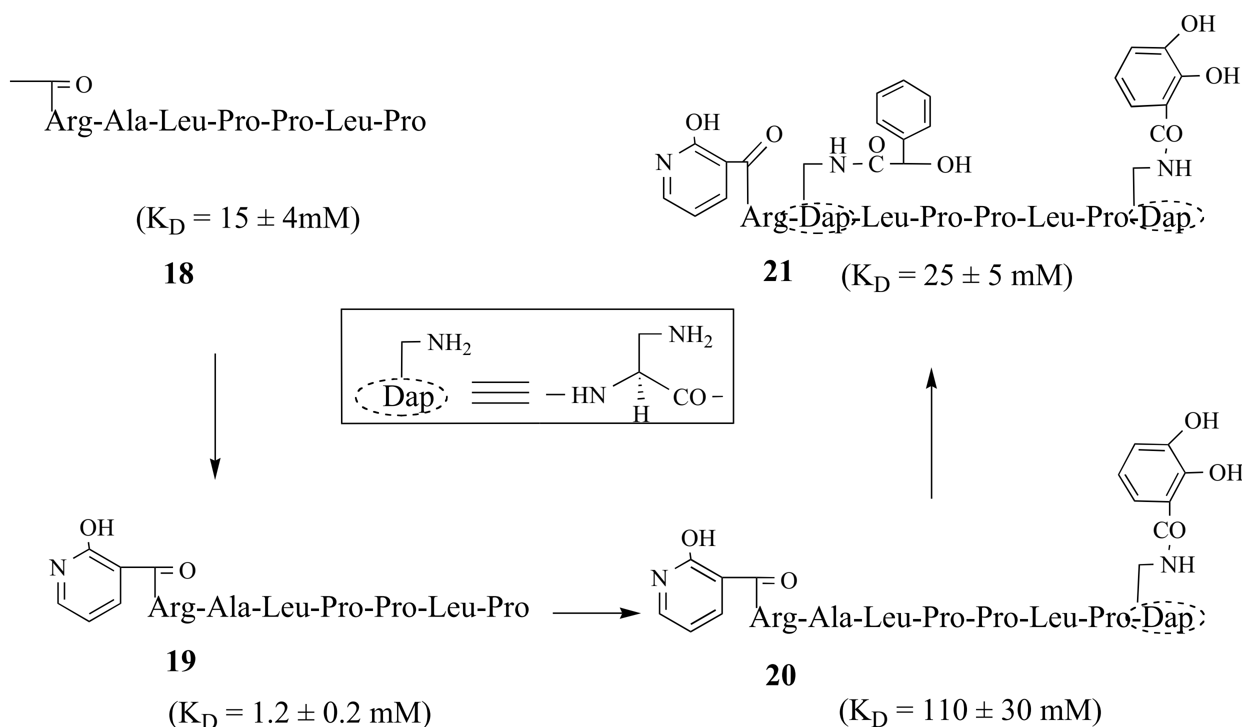


Fig. (3).

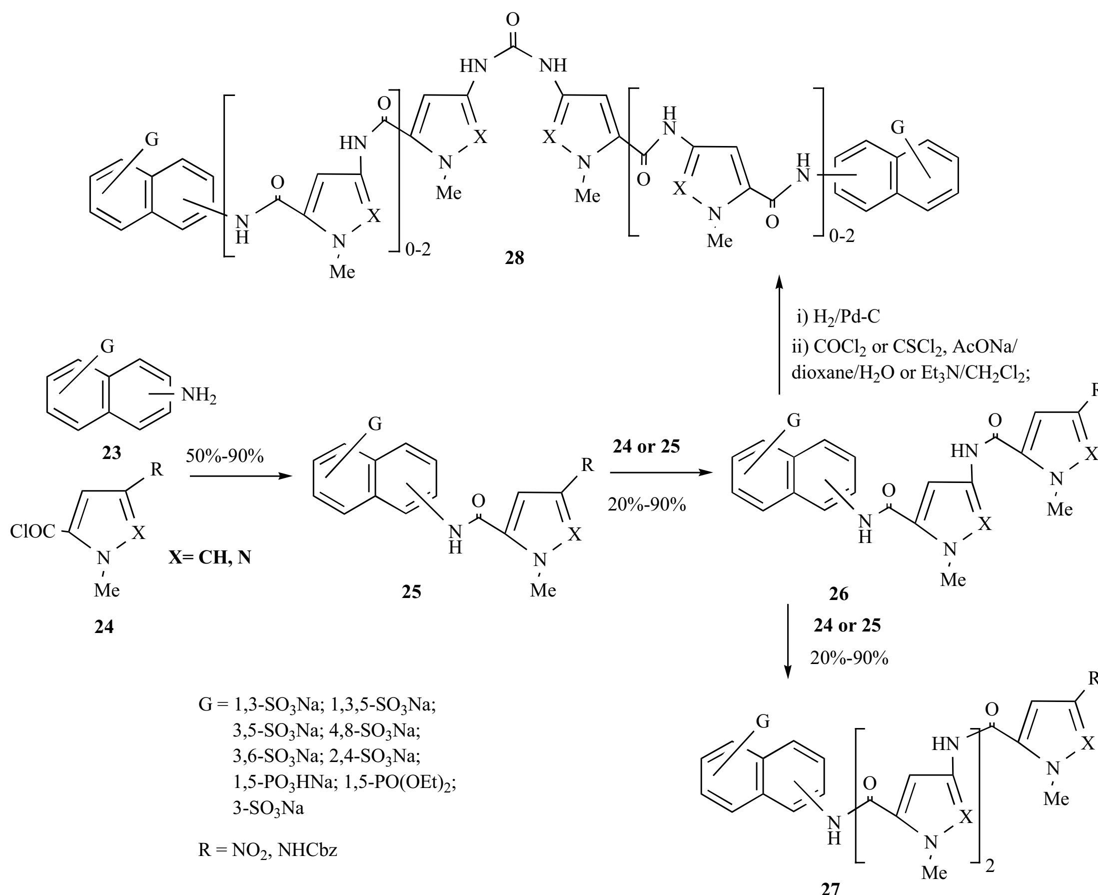
trypanosomiasis (sleeping sickness) and onchocerciasis since the early 1920s [48]. It was originally synthesized by Bayer AG in 1916 based on the observation that trypan red and trypan blue exhibited trypanocidal activity [49]. This com-



Scheme 1.

pound is known to inhibit a large number of important enzymes [50] and to block the activity of several growth factors, one of which is bFGF [51]. Given the structural and chemical nature of suramin, it lacks a reactive functionality and possesses six sulfonic acid groups attached directly to aromatic rings. This functionality made them binding with protein-tyrosine kinases and protein tyrosine phosphatases that control the state of tyrosine phosphorylation in cellular proteins, which regulate a wide variety of biological processes such as cell growth, differentiation and oncogenic transformation [52]. Since the growth factors inhibited by suramin are primarily heparin binding proteins, it is likely that the interaction of suramin with these proteins involves primarily their polyanion binding sites [53]. In fact, heparin disrupts the FGF-1-suramin complex [54], and counteracts the antiangiogenic effect of suramin [55]. The clinical utility of suramin is hampered by its unfavorable toxicity profile (important adverse neurotoxic side effects) and its narrow therapeutic index. Toxicity is related to extremely long plasma half-life (45-55 days) and great metabolic stability of suramin [56] leading to high plasma concentrations of this compound (the prolonged half-life is a consequence of being tightly bound to serum proteins, mainly albumin [57]). As a consequence, monitoring of plasma concentration of suramin has become a step of therapeutical protocols [58]. However, toxicity decreased by using suramin in combination with chemotherapy (vinblastine [59] or epidoxorubicin [60]) allowing reduced doses of suramin and improved therapeutic efficacy [61].

Designing novel compounds able to complex bFGF and consequently block the angiogenic process more effectively and lower toxicity than suramin was the interest of many labs [62]. As shown in Scheme **2**, a series of pyrrole and pyrazole congeners of suramin were prepared [62b] by acylation of sulfonated or phosphonated naphthylamine **23** with 1-methyl- 4-nitropyrrole-2-carbonyl chloride or 3-benzyloxycarbonylamino-1-methylpyrazole-5-carbonyl chloride **24** to give amides **25**, followed by reduction to the corresponding aminoamides by catalytic hydrogenation. Starting from aminoamides derived from **25**, these reactions were repeated once or twice to furnish the corresponding amines **26** and **27**, respectively, through corresponding nitro derivatives intermediates. Catalytic reduction of **26** followed by treatment of the corresponding amines with phosgene in the presence of sodium acetate afforded the final ureido derivatives **28**. The overall yield of the whole preparation is ranging between 17 and 60%, depending on the number of heterocyclic spacers introduced. All these compounds **28** (generally called suradistas) contain the amido-*N*-methylpyrrolnaphthalenesulfonic acid group, but differ in the number of *N*-methylpyrrole groups as well as in the number and position of sulfonic acid residues on the naphthalene rings.



Scheme 2.

Derivatives of compound **28** were evaluated for their ability to inhibit bFGF binding *in vivo* bFGF-induced angiogenesis and neovascularization of the chorioallantoic membrane (CAM) in comparison with suramin (Table **2**). Most of the tested compounds showed moderate to good activity in the given assays. Both the number of the sulfonate groups and their position relative to the amino moiety on the naphthalene rings of have a little influence on the biological properties, whereas the length of the molecule seems to be a crucial factor: while good activity is still exhibited by the longer molecule (n = 2). The substitution of pyrazole for pyrrole ring does not positively affect the biological profile, in terms of either activity or toxicity.

In a study using a proposed binding model to better understand the nature of interaction between bFGF and the antiangiogenic drug (**28**, entry 1, Table **2**); 7,7-(Carbonyl-bis [imino-*N*-Methyl-4,2-pyrrolecarbonylimino[*N*-methyl-4,2-pyr-role]-carbonylimino])-bis-(1,3-naphthalene disulfonate); reported by Zamai *et al.* [62a] revealed that the binding to the protein is driven by the combined hydrophobic and hydrogen bonding acceptor sites of the pyrrole backbone. It was further strengthened by local and specific interaction of sulfonic charged groups. Specificity within the proposed model in this study was demonstrated not only by the solvent effect, but also by the studies of point chemical modification. Results showed that the ligand tight but reversible binding to bFGF in a 1:1 complex. It is most likely that this interaction covers a large surface of the protein involving the heparin-binding domain and the selenate-binding site. Thus this relatively small molecule, forming a 1:1 complex with bFGF, is able to prevent biologically effective dimerization of bFGF.

The mechanism of the biological activity of suramin, suramin derivatives and suradistas is still unknown [63]. Their inhibition of mitogenic and angiogenic activity of FGFs, and

Table 2. Biological Data of Compounds 28 in Comparison to Suramin22

NH Pos.	X	Y	W	n	Inhibition of bFGF Binding (ID_{50}, μM)[a]	Inhibition of bFGF- Induced Angiogenesis (%)[b]	Inhibition of CAM Vascularization (%)
7	CH	CH	1,3-SO3Na	1	142 ± 18	100	100
7	CH	CH	1,3,5-SO3Na	1	116 ± 10	93	80
7	CH	CH	1,3-SO3Na	2	163 ± 16	89	62
8	CH	CH	1,3-SO3Na	1	390 ± 34	82	77
8	CH	CH	1,3,5-SO3Na	1	153 ± 15	80	60
7	CH	CH	1,3-SO3Na	1	135 ± 11	38	100
7	CH	CH	1,3-SO3Na	0	550 ± 92	ND[d]	30
7	CH	CH	3,5-SO3Na	1	085 ± 18	100	83
8	CH	CH	3,5-SO3Na	1	109 ± 12	90	71
7	CH	CH	4,8-SO3Na	1	145 ± 17	94	67
7	CH	CH	3,6-SO3Na	1	146 ± 6	0	63
7	N	N	1,3-SO3Na	1	171 ± 14	ND	9
7	CH	CH	2,4-SO3Na	1	183 ± 35	ND	83
7	CH	CH	1,5-PO3HNa	1	285 ± 40	ND	Tox[e]
8	CH	CH	1,5-SO3Na	1	587 ± 20	100	65
7	CH	N	1,3-SO3Na	1	345 ± 20	0	Tox
7	N	CH	1,3-SO3Na	1	677 ± 12	ND	44
8	CH	CH	3-SO3Na	1	857 ± 109	0	45
Suramin					091 ± 11	94	87

[a] ID_{50}: dose inhibiting by 50% the binding of bFGF to its receptor determined on BALB 3T3 cells after 4 h incubation in the presence of 0.2nM ^{125}I-bFGF and scalar concentrations of each compound.

[b] Inhibition of in vivo angiogenesis induced by bFGF gelfoam implanted s.c. in C3H/He mice treated 24 h later with 200 mg/kg iv of each compound. The degree of angiogenesis was measured by counting the number of vessels in control and treated mice after 15 days implantation.

[c] Inhibition of angiogenesis on the chorioallantoic membrane (CAM) assay. Results are reported as percentage of CAMs presenting an avascular zone with at least 2mm in diameter after 48 h treatment with 350 nmol of each compound incorporated into a methylcellulose pellet.

[d] ND=Not determined.

[e] Tox=toxic at the tested dose.

the important role of sulfates moieties of heparin in its interaction with FGFs, suggest a key role for naphthalenesulfonate groups in the inhibition process. Thus, the mechanism of action was suggested to involve their direct binding to the growth factor *via* the polyanionic binding sites.

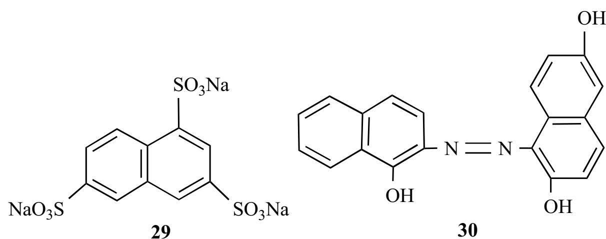


Fig. (4).

To support this hypothesis, 1,3,6-naphthalenetrisulfonate **29**, Fig. **4**, the common chemical feature of suramin and suradista families, has been shown to effectively inhibit the mitogenic activity of FGF-1 [64], and suppress the FGF-1-dependent angiogenesis [65] by direct interaction with the growth factor. In a similar way, compounds structurally characterized by naphthalensulfonic moieties, such as Eriochrome Black T **30**, are potent inhibitor of angiogenesis and tumor growth *in vivo* and exhibit diminished toxicity with respect to suramin **22** [66], underscoring the importance of sulfonic acid substituted naphthalenes in determining antiangiogenic and antitumor activity. On these bases, some conclusions about the structural features for such inhibitors antiangiogenic can be drawn: i) two moieties bearing acid groups (e.g. naphthalenesulfonic moiety) at a defined distance are essential, with the symmetric molecules more active than the asymmetric ones [67] bearing only one acid moiety (e.g. only one naphthalenesulfonic substituent). The distance between the acid groups (e.g. size and number of spacers) is important for antiangiogenic activity. ii) Rigid and flat compounds, structurally similar to suramin, have been shown as active as or more active than suramin itself. iii) The presence of flexible bridge that allows many conformations of the molecule is not the optimal spacer between the acid groups.

2.3. Sulfonamide Derivatives as a Unique Angiogenesis Inhibitor

Angiogenesis *i.e.,* the formation of new blood vessels, results from a series of molecular events that are regulated by stimulators (positive regulators) and inhibitors (negative regulators) [68] leading to the stimulation of endothelial cells which leave their resting state and start to digest the basement membrane, proliferate, migrate and differentiate to form a hollow tube. All these steps can be induced by growth factors able to regulate endothelial cells. In particular, FGF-1 and FGF-2 are the most extensively studied positive modulators of new blood vessel formation, together with vascular endothelial growth factor (VEGF) [69], and platelet-derived growth factor (PDGF) that appear to be the most potent. Because angiogenesis involves multiple systems *in vivo*, the possibility that tolerance to an angiogenesis inhibitor might develop must be considered. Table **3** compiles the main characteristic polypeptide growth factors and their receptors.

Therefore, novel types of angiogenesis inhibitors might be useful to decrease the risk of resistance developing to other angiogenesis inhibitors.

Based on the fact that aromatic sulfonamide derivatives exhibit a range of bioactivities such as antimicrobial [71], antidiabetic [72], anti-inflammatory [73], and anticancer [74-76]. It has been reported the discovery of a novel angiogenesis inhibitor E 7820; *N*-(3-Cyano-4-methyl-1H-indol-7-yl)-3-cyanobenzene-sulfonamide **31** (Fig. **5**) among sulfonamide derivatives and used a TF model for screening assay. Results revealed that the novel antiangiogenic sulfonamide derivative modulates the expression of integrin α 2, 3, 5, and β1 on

Table 3. Main Characteristic Polypeptide Growth Factors and their Receptors [70]

Ligand Receptor	Length (Aminoacids)	MWt (kDa)	Human Chromosomal Location
aFGF	115	16	4
bFGF	155	16-18	5
PDGF	110-125 (A)	14-18	7
VEGF-1	121	-------	6
VEGF-2	165	-------	6
FGF-R-1	801	160	8
FGF-R-2	800	135	10
PDGF-R	1066 (a)	170	4
	1074 (b)	180	5
Flt-1	1273	180	13
Flk-1/KDR	1367	230	5

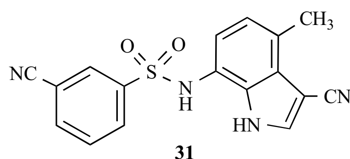


Fig. (5).

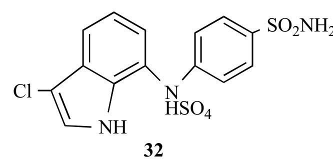


Fig. (6).

HUVEC [77]. Interestingly, oral administration of E7820 **31** remarkably resulted in inhibition of tumor-induced angiogenesis in mouse dorsal air sac model, and tumor growth of human colorectal tumor cell lines (WiDr and LoVo) was inhibited in xenotransplanted model in mice. This discovery of novel small molecule that is able to modulate integrins may provide the basis for a new approach to antiangiogenic therapy through the suppression of integrin α2 on endothelium.

(E7070) **32** showed significant antitumor activity against HCT116 human colon carcinoma both *in vitro* (IC_{50} 0.11 μg/mL in cell proliferation assay) and *in vivo* (not only growth suppression but also a marked reduction of tumor size in three colon cancers (HCT116, SW620 and HCT15) and two lung cancers (LX-1 and PC-9); (Table **5**). (E7070) **32** is currently undergoing phase I clinical trials in European countries.

Table 4. Antiangiogenic Activity of E7820, SU-5416 and Marimastat *In Vitro*

Assay	Angiogenic Factor Stimulation	E7820	IC_{50} (μg/ml)		Marimastat
			TNP-470	SU-5416	
Rat Aorta angiogenesis	--------	0.11	0.00049	0.17	0.34
Sandwich TF assay	bFGF	0.20	>5	>5	>5
	VEGF	0.24	>5	0.19	>5
Cell growth assay (HUVEC)					
10% FCS	BBEa	0.33	0.0003	3.4	21
Serum-free	bFGF	0.1	8.5	0.36	ND
	VEGF	0.081	10	0.023	ND

a. BBE. Bovine brain extract. ND, not done

The idea of tumor-induced angiogenesis (i.e. tumor- induced growth of new vessels) was first introduced by *Virchow* in 1863 [78]. Although initially this concept was sceptically considered in specialist circles, it was periodically repeated by several scientists, until the *Folkman's* group reported in 1971 the isolation of a tumor factor responsible for angiogenesis [79]. In 1985 *Vallee's* group succeeded in purifying to homogeneity angiogenin, an angiogenic protein derived from human colon adenocarcinoma [80]. In the last 10 years, a wealth of angiogenic factors produced either by tumor or by normal tissues have been described [81] and it is now widely accepted that angiogenesis is a fundamental process in the development, progression and metastasis of many human tumors, in that it allows the blood supply necessary for tumor growth [82]. Owa *et al.* [75] reported the discovery of a novel series of antitumor (Fig. **6**) sulfonamides targeting G1 phase of the cell cycle. Cell cycle control in G1 phase has attracted considerable attention in cancer research, because many of the important proteins involved in G1 progression or G1/S transition have been found to play a crucial role in proliferation, differentiation, transformation, and programmed cell death (apoptosis). Of the compounds examined, *N*-(3-chloro-7-indolyl)-1,4-benzenedisulfonamide

Watanabe and others [76,77] reported the synthesis of *N*-(2-anilino-3-pyridy1) benzenesulfonamide analogs **38** as novel types of active sulfonamides antitumor agents. The syntheses of these analogs are outlined in Scheme **3**. Thus, heating the mixture of 2-chloro-3-nitropyridine **33** and aniline **34** or its derivatives at 100 ºC furnished the corresponding 2-anilino-3-nitropyridine derivatives. Catalytic hydrogenation and condensation with *p*-substituted benzene sulfonyl chlorides **37** afforded the target analogues **38**.

2.4. Glycosides and Spaced Sugars

The anticoagulant activity of heparin pentasaccharide sequence [83] prompted synthetic efforts aimed at the procurement of this structure as well as a host of related sequences **39**, Fig. **7**. The synthetic heparin oligosaccharide **39a** [83,84] and α-methyl-glycoside analog **39b** [85,86] displaying the same biological properties. The synthesis of analogs of **39a** has been reviewed in detail [87]. Pentasaccharides **39c**–**39g** were synthesized to establish a detailed structure-activity relationship. Replacement of the reducing end glucosamine by a glucose residue indicated that *O*-sulfates are effective substitutes for *N*-sulfates [86]. Introduction of an extra 3-*O*-sulfate group at the reducing end of pentasac-

Table 5. Antitumour Activity of E7070 in Various Human Tumour Xenograft Models on Daily Intravenous (i.v.) Administration for 4 days (QDx4)

Xenograft model	Dose (mg/kg/day)	RTV_{min} [a]	RBW_{min} [b]	T/C value (%)	Dead[c]/treated
HCT116	12.5	0.81	–	34	0/5
colon	25	0.44	–	23	0/5
	50	0.15	0.93	8	0/5
LX-1	12.5	–	0.96	80	0/5
lung	25	0.79	–	29	0/5
	50	0.27	0.95	2911	0/5
SW620	12.5	–	–	88	0/5
colon	25	–	–	67	0/5
	50	0.63	0.91	34	0/5
	100	–	0.89	–[d]	5/5
HCT15	25	–	0.98	74	0/5
colon	50	0.46	0.88	26	0/5
PC9	12.5	–	–	89	0/5
lung	25	–	0.99	83	1/5
	50	0.93	0.95	35	0/5
	100	0.89	0.84	–[d]	5/5
DLD-1	25	–	–	83	0/5
colon	50	–	0.79	54	0/5
WiDr	12.5	–	–	85	0/5
colon	25	–	–	77	0/5
	50	–	0.95	72	0/5
	100	–	0.88	–[d]	5/5

At the termination of the experiments, the average of tumour weights ± standard deviation (S.D.) of the vehicle control groups were: HCT116, 1.53 ± 0.50; LX-1, 1.72 ± 0.68; SW620, 1.18 ± 0.33; HCT15, 1.21 ± 0.37; DLD-1, 0.56 ± 0.25; PC9, 0.47 ± 0.15; WiDr, 0.73 ± 0.19 g.

a Minimum relative tumour volume; – means no reduction of tumour volume was observed.

b Minimum relative body weight; – means no body weight loss was observed. The RBWmins of vehicle control group in the HCT116, LX-1, HCT15 and DLD-1 xenograft models were 0.97, 0.81, 0.97 and 0.99, respectively. In control group in the SW620, PC9 and WiDr, the body weight loss was not observed.

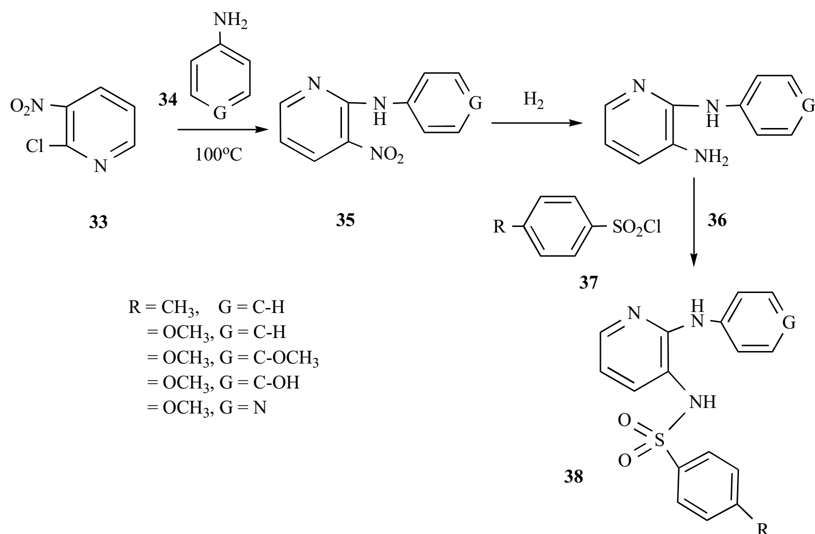
c Number of dead mice during the experiment.

d Tumour weight was not available because all mice were dead.

charide **39c** increased factor activation of AT III Xa affinity [88]. Partial and complete *O*-methylation (**39b**–**39g**) did not significantly alter AT III affinity [89,90], Table **6**.

Analogs **40a**–**40f** closely resemble heparin oligosaccharides and their functions but are simpler to synthesize. These so-called "nonglycosamino" glycans contain only *O*-sulfate esters and *O*-alkyl ethers [91]. All hydroxyl groups are permanently capped as methyl ethers, thus eliminating the need to discriminate between nonsulfated and sulfated hydroxyl groups. Many analogs with pseudo-alternating sequences have been synthesized [92,93]. Pentasaccharides **40a**–**40c** contain a common tetrasaccharide composed of 2-*O*-sulfate and 3-*O*-methyl uronic acid moieties, whereas pentasaccharides **40d**–**40f** contain an invariable tetrasaccharide composed of 2,3- di-*O*-methyl uronic acid moieties, Fig. **7**. Pentasaccharide **40d**, containing not less than seven methyl ethers, displays the highest anti-Xa activity (1611 units/ mg), Table **7**, and is the most potent analog of 31 identified to date.

Sulfated pentasaccharide connected to different spacers (charged, neutral, linear, flexible or rigid) were prepared in order to obtain heparin-like oligosaccharides with full anticoagulant properties [94,95]. Figure **8** compiles some reported examples derived from pentasaccharide **41** together with their biological activities. Based on the observation that oligonucleotides can associate with the heparin binding site



Scheme 3.

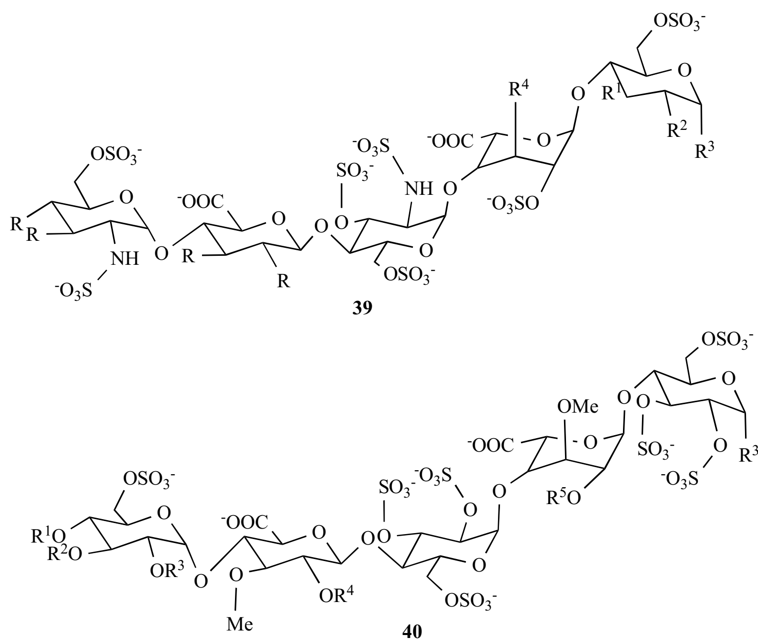


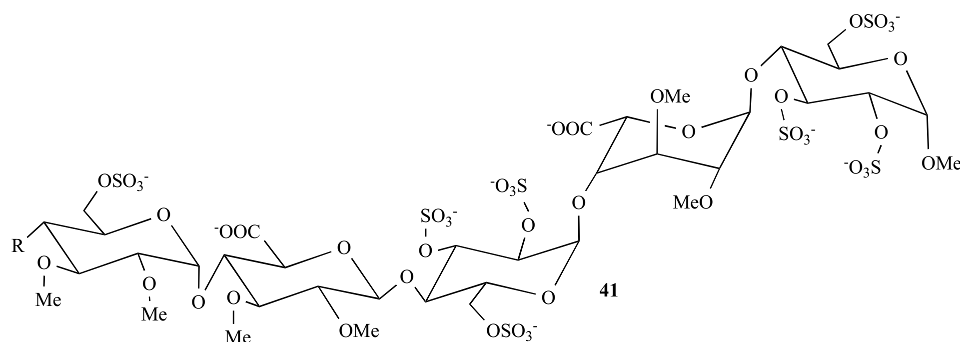
Fig. (7).

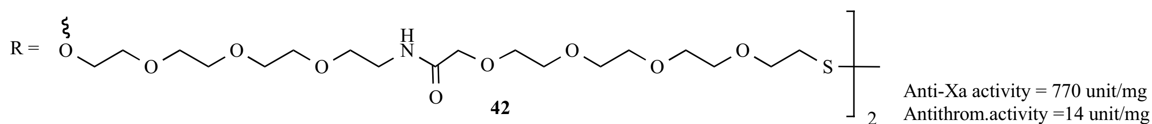
Table 6.

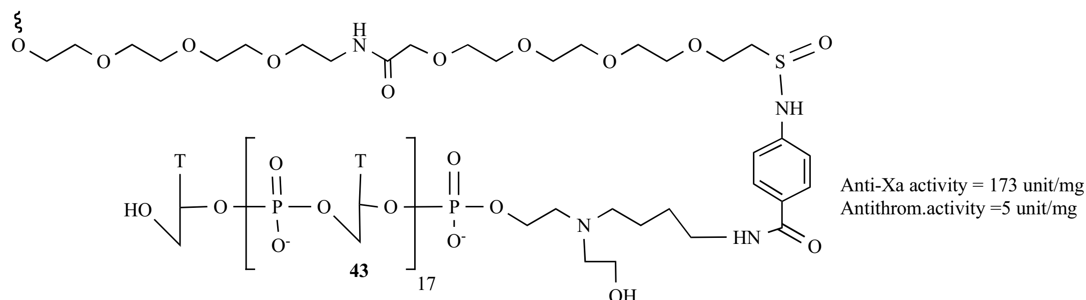
Cpd	R	R[1]	R[2]	R[3]	R[4]	Anti-Xa Activity
39a	OH	OH	$NHSO_3^-$	OH	OH	700 mg/unit
39b	OH	OH	$NHSO_3^-$	OMe	OH	700 mg/unit
39c	OH	OSO_3^-	$NHSO_3^-$	OMe	OH	1270 mg/unit
39d	OH	OSO_3^-	OSO_3^-	OMe	OH	1300 mg/unit
39e	OH	OSO_3^-	OSO_3^-	OMe	OMe	1110 mg/unit
39f	OMe	OSO_3^-	$NHSO_3^-$	OMe	OH	1288 mg/unit
39g	OMe	OSO_3^-	OSO_3^-	OMe	OMe	1323 mg/unit

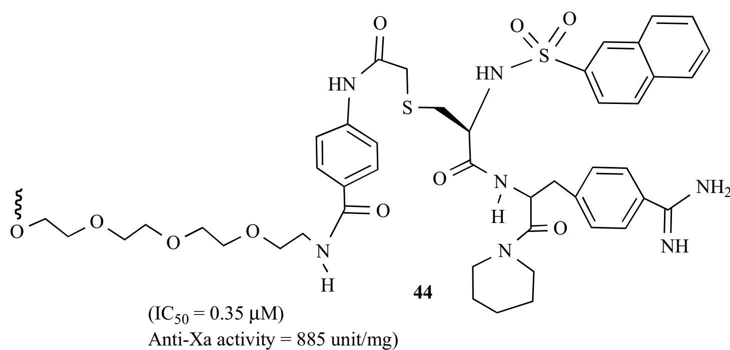
Table 7.

Cpd	R^1	R^2	R^3	R^4	R^5	Anti-Xa Activity
40a	Me	Me	Me	SO_3^-	SO_3^-	1217 mg/unit
40b	Me	Me	SO_3^-	SO_3^-	SO_3^-	1159 mg/unit
40c	Me	SO_3^-	SO_3^-	SO_3^-	SO_3^-	1184 mg/unit
40d	Me	Me	Me^-	Me	Me	1611 mg/unit
40e	Me	Me	SO_3^-	Me	Me	1318 mg/unit
40f	Me	SO_3^-	SO_3^-	Me	Me	1404 mg/unit









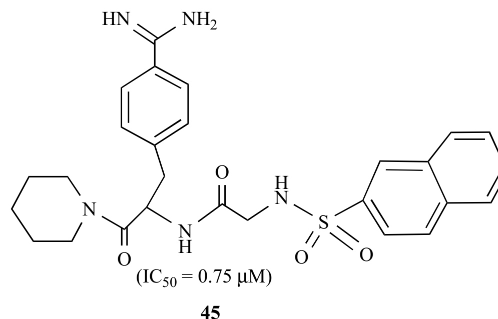
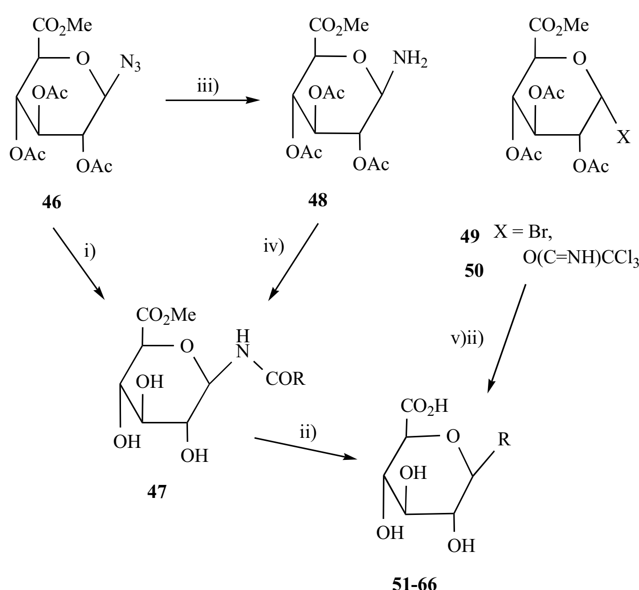


Fig. (8).

[96], conjugate **43** was prepared in which part of the spacer were replaced by an oligonucleotide. The low anti-factor Xa activity (173 units/ mg) and antithrombin activity (5 units/ mg) of **43** illustrated the existence of a weak interaction of oligonucleotides with the TBD domain. *N*-(2-Naphthalene-sulfonyl)-glycyl-(D)-4-aminophenyl alanyl-piperidine (NAPAP) derivate **44** [97] exhibited antithrombin activity (IC_{50} = 0.35 μM) and antifactor Xa activity (885 units/mg) and confirmed that the NAPAP conjugate is a better inhibitor than the combination of the free pentasaccharide and NAPAP. NAPAP **45** itself binds directly to the active site of thrombin (IC_{50} = 0.75 μM).

Hindsgaul and co-workers [98] have shown that evaluation of *carbohybrid* libraries composed of D-galactopyranose that carry a diverse range of small non-carbohydrate aglycon structures led to the identification of μM inhibitors of a galactose binding plant lectin. This report was the turning point for investigating strategies for the design or identification of novel, small molecule inhibitors, which are reduced in carbohydrate character, of these FGF mediated signaling pathways. Murphy *et al.* [99] reported the identification of novel glucuronic acid derivatives that inhibited heparin binding to FGF-2 in enzyme linked immunosorbant assays (ELISAs). These compounds also inhibited endothelial cell survival



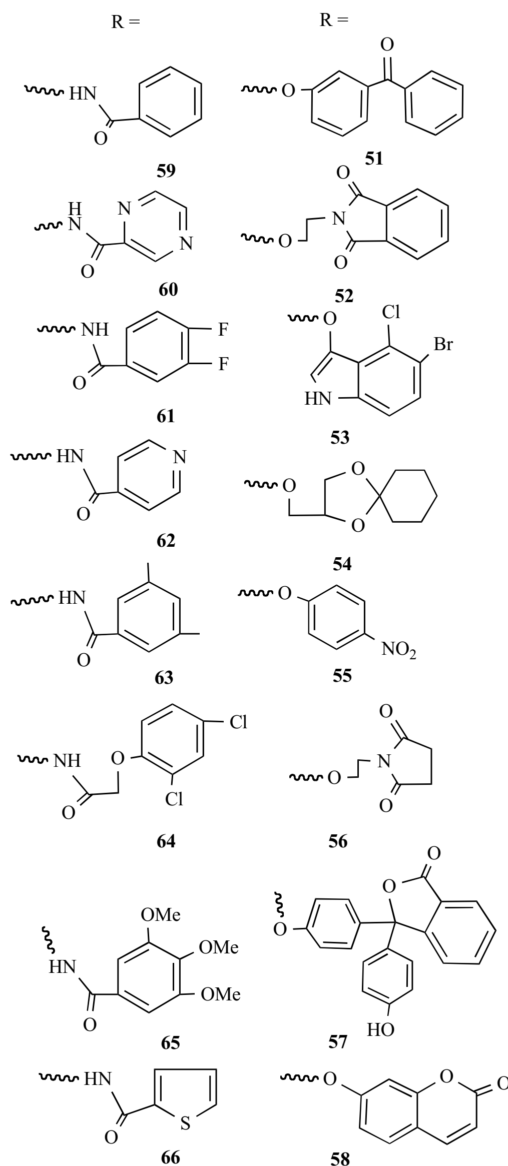


Table 8. Inhibition of Binding of Heparin–Albumin to FGF-2 Determined by ELISA[a]

Compd	*I*max[b] (%)	IC_{50} (ng/mL)
Heparin–albumin	95	0.6
Heparan sulfate	70	136.5
Heparin	95	1.61
56	25	<100 (340 nM)
61	39	11,000
62	45 at 106 ng/mL	—
64	24	10
65	23	58
66	40 at 106 ng/mL	—

a All other compounds were not inhibitors.

b IC50 is defined as concentration of carbohydrate required to obtain 50% of maximum inhibition (*I*max).

Key: (i) RCOCl, CH_3CN then Ph_3P or diphenylphosphinopolystyrene; (ii) LiOH, H_2O, THF, MeOH; (iii) Pd/C, H_2, -15 °C, THF, 2 h; (iv) RCO_2H, DCC, HOBT, DMAP, THF; (v) $AgCO_3$, $AgClO_4$, MS 4°A, dry CH_2Cl_2, ROH; (vi) BF_3OEt_2, CH_2Cl_2, ROH.

Scheme 4.

pathways that depend on interactions of heparan sulfate proteoglycans, FGF-2 and FGFR. The readily available glucuronic acid was chosen for this invistigation as it is one of the simplest, active and charged, saccharides found in heparin structures. The synthesis of these *O*- and *N*-glycosides inhibitors **51-66** and biological evaluation are outlined in Scheme **4** and Table **8**, respectively. Results indicated that compounds **56**, **61-62**, **64-66** showed inhibitory activity in the ELISA assay and also inhibit cell survival. Other compounds neither showed inhibition in the binding assay nor altered cell viability. Compounds **62** and **66** inhibited survival activity of the endothelial cells at the concentrations used (~33 µM) through their inhibition of the interaction of heparan sulfate proteoglycans with FGF-2.

Spaced fully sulfated disaccharides in which two units of disaccharide units are separated by aromatic spacer **67-68** and C6 open chain sugars attached by various linkers to the aromatic spacer **69-72**, Fig. **9**, have been reported to have a remarkable antiproliferative activity significantly higher than

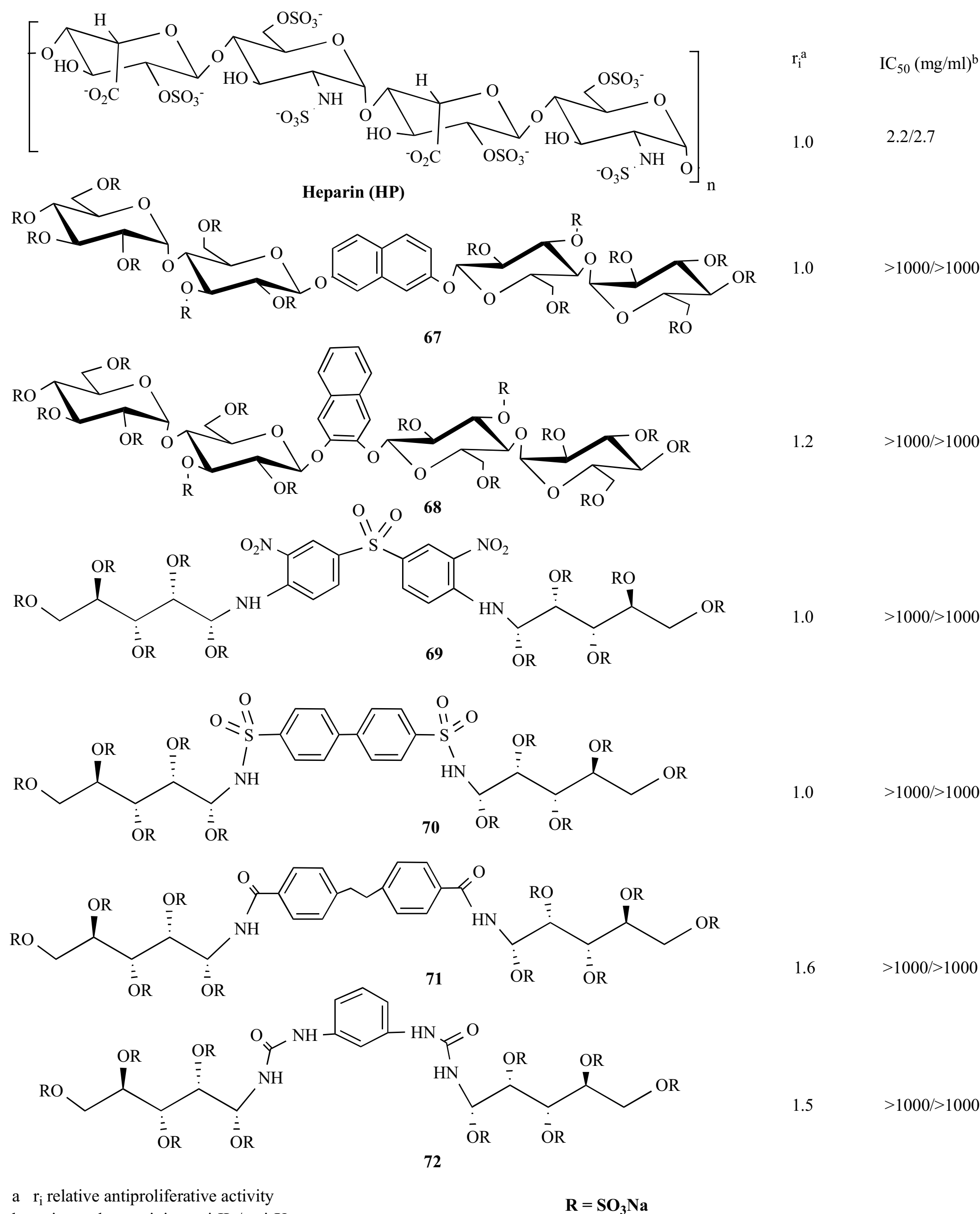


Fig. (9).

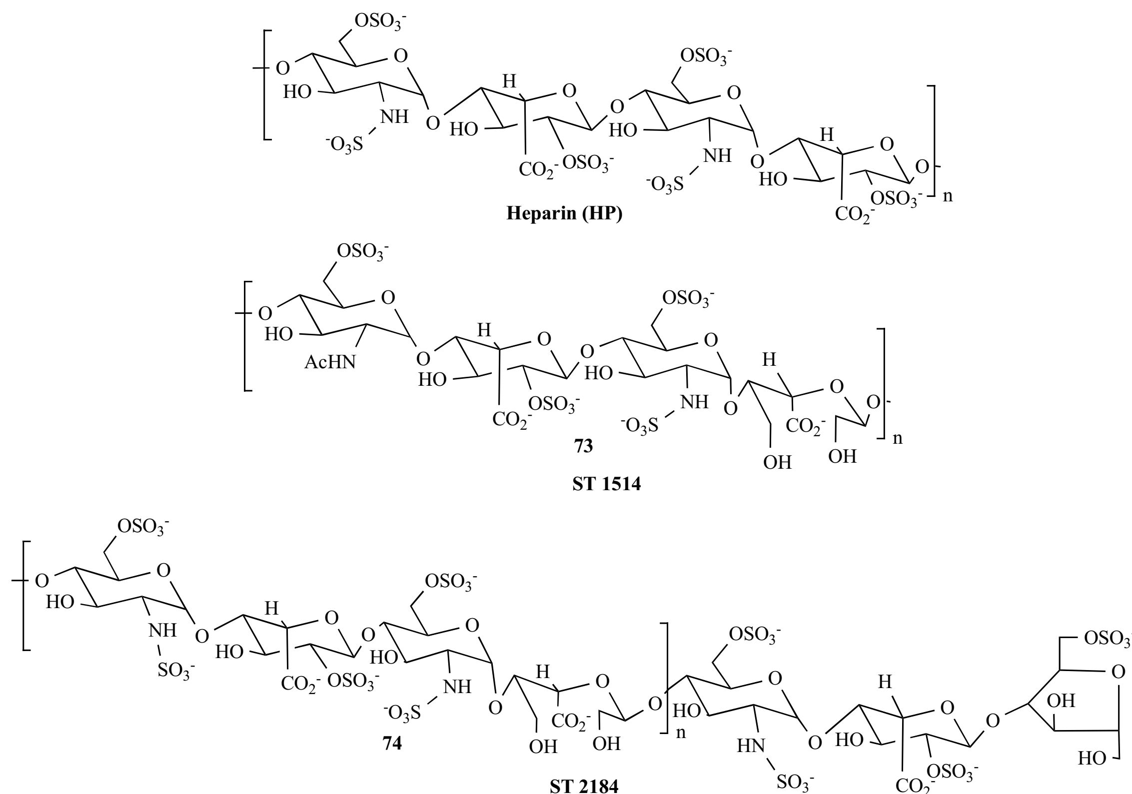


Fig. (10).

heparin, which points at the contribution of the spacer to the overall binding [100,101]. The synthesis of the open chain spacer is straightforward starting from 1-amnio-1-deoxy-glucitol and reaction with dicarboxylic acids followed by sulfation afforded the amide linkage open chain spacers.

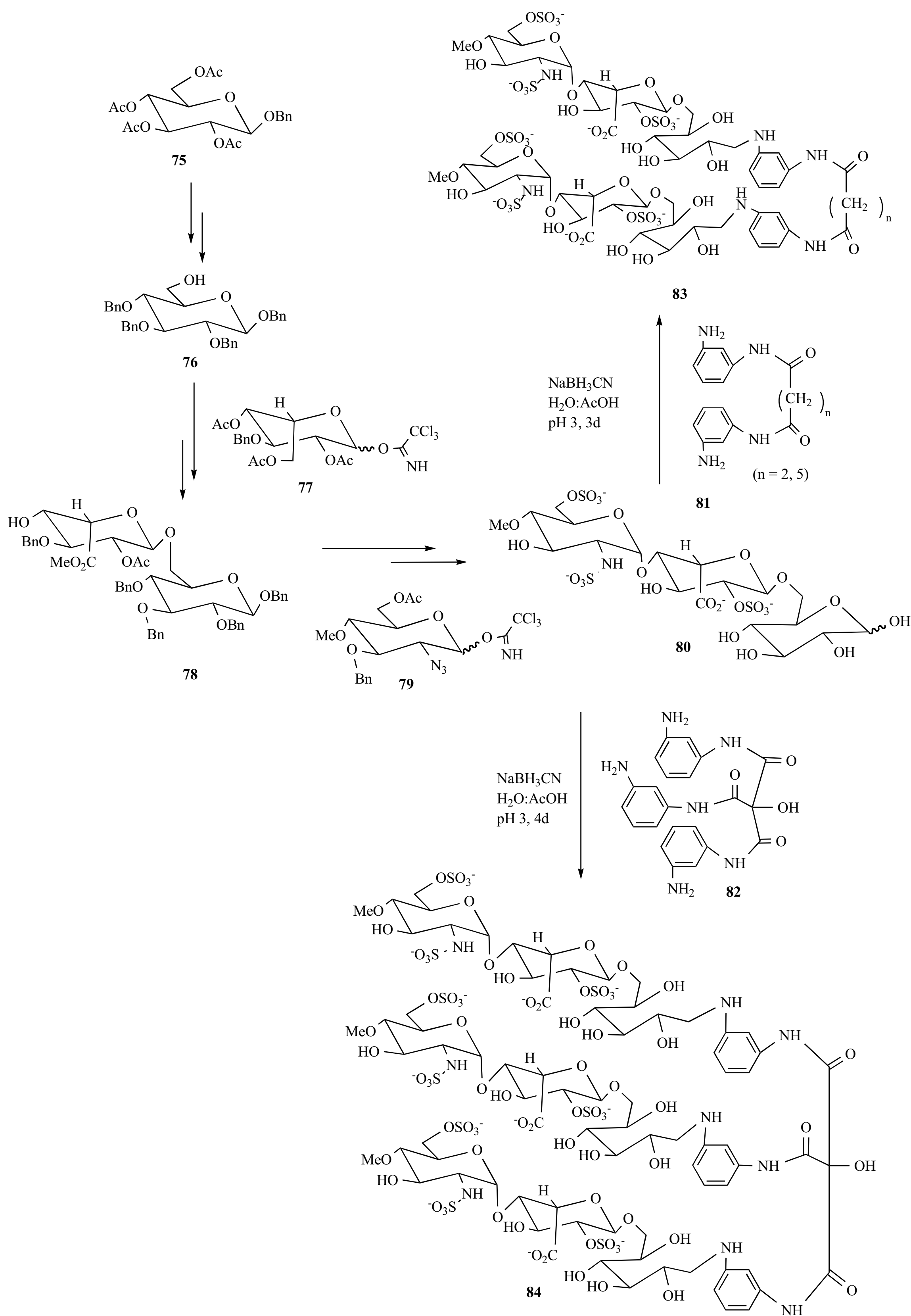
Heparin is used in therapy as an anticoagulant and antithrombotic drug [102], however, the anticoagulant properties of heparin involve hemorrhagic risks. When administered to cancer patients, heparin increases survival times [103] but the hemorrhagic risks and non-anticoagulant variants of the polysaccharide endowed with potential antitumor properties are warranted [104].

Casu *et al.* [105], generated a novel nonanticoagulant antiangiogenic heparin derivative **73** (ST1514) (Fig. **10**) by a two-step modification of unfractionated heparin sample: i) sulfation gaps along the regular heparin sequences were generated by selectively removing 2-O-sulfate groups to reach a ratio of about 1:1 between sulfated and nonsulfated uronic acid residues; ii) the C(2)–C(3) bonds of all nonsulfated uronic acid residues were split, generating flexible joints along the heparin chain while minimizing cleavage of glycosidic bonds. Because the splitting reaction also occurs at the level of the essential glucuronic acid residue of the active site for antithrombin, ST1514 was no longer anticoagulant, but it showed a potent FGF2 antagonist and angiostatic activity. These results attracted the attention to design low-molecular weight (LMW) nonanticoagulant antiangiogenic substances exploiting binding to VEGF while preventing receptor engagement. (LMW) heparin inhibits the angiogenic activity exerted by $VEGF_{165}$ [106]. Recently, a (LMW) compound (**74**, ST2184, Mw = 5800) generated by depolymerization of an undersulfated glycol-split heparin derivative has been reported [107]. Biological assays proved that ST2184 interacts with $VEGF_{165}$ with an affinity (half at doses ID_{50}) ~10 times higher than heparin but 3 times lower than LMW heparin, Table **9**. Compared to heparin, it has a negligible anticoagulant activity providing the basis for the design of novel nonanticoagulant antiangiogenic compounds.

Table 9. inhibition of Immobilized Heparin/$VEGF_{165}$ Interaction (ID_{50} (nM)

Heparin	10
ST2184	100
LMWH	300

Heparin binds to platelets and can cause activation and aggregation. The platelet binding site for heparin has to be determined to understand how heparin is platelet function. Given the heterogeneous nature of heparin, this is a challenging task. A disaccharide unit (GlcNS[6-OS]-IdoA[2-OS]) in heparin was found to be the key for the binding interaction [108]. The work reported by Koshida *et al.* [109, 110] for the efficient preparation of complex and diverse types of oligomeric compounds **83**, **84**, Scheme **5**, was devised to assemble components containing the 'completed' GlcNS6S-IdoA2S unit already sulfated and deprotected. The key



Scheme 5.

sulfated trisaccharide component **80** was employed for many reasons; a) it is composed of the GlcNS6SIdoA2S disaccharide unit and an additional D-glucose; b) Using the reducing end of the glucose moiety, the key component can be assembled with aromatic amino groups of linker molecules in one-step by the reductive amination method; c) The glucose unit works not only as a reducing end donor for the reductive amination, but also as the hydrophilic portion in the molecule to minimize any non-specific hydrophobic interactions between the linker and the target proteins or cells. The key trisaccharide was prepared starting from the known benzyl β-D-glucopyranoside **75** that converted to benzyl 2,3,4-tri-*O*-benzyl-β-D-glucopyranoside **76** in four-step reaction. The latter was then coupled with the known L-idose trichloroacetimidate derivative **77** to give the desired a-linked disaccharide that is converted to **78** by standard four steps chemistry. The 2-azido sugar derivative **79** was coupled with the disaccharide **78** to give selectively an α-linked trisaccharide followed by de-*O*-acetylation, sulfation, and reduction of the azido function to give **80**. Coupling with linkers **81** and **82** dimeric assembly **83**, possessing methylene bridge with different length, and the trimeric assembly **84** possessing three units of GlcNS6S-IdoA2S.

Platelet binding activities were determined and results revealed that compounds which contain three units of GlcNS (6-OS)-IdoA(2-OS), bound stronger than their counterparts which contain only two key disaccharides. These findings underscore the role of the GlcNS(6-OS)-IdoA(2-OS) clustering effect for binding. The binding potency is not influenced by the distance between the GlcNS(6-OS)-IdoA(2-OS) units Head-to-tail dimer has a higher binding activity than the tail-to-tail dimer. The arrangement of the two units of GlcNS(6-OS)-IdoA(2-OS) has an influence on the activity.

2.5. Non-Peptide, Non-saccharides Inhibitors

Potential therapeutic approaches to inhibit angiogenesis include neutralizing antibodies against VEGF, soluble receptors, ribozymes directed against VEGF receptor, and VEGFR tyrosine kinase inhibitors that target the intracellular signal transduction. A variety of anti-angiogenesis therapies directed against the VEGFR kinase have been a promising and well-validated therapeutic approach under active evaluation of their safety and efficacy in multiple clinical trials. [111]. In a recent study reported by Pattabiraman and others [112] to develop a pharmacophore based on the binding of ATP to the hinge region of the kinase domain of VEGFR and a database search of 18,000 compounds was conducted.

Two sets of compounds **85-87** and **88-90** representing (1,3-dioxo-2,3-dihydro-1H-isoindol-4-yl)-amide and (1,3-dioxo-2,3-dihydro-1H-isoindol-5-yl)-amide were taken up on the basis of the binding features and tested for angiogenesis activity (Fig. **11**). Compounds **91** and **92** were chosen with a representative substitution pattern due to their viability for multiple hydrogen bonds with the protein. Selected hits were assessed for their ability to limit the induction of web-like network of capillary tubes by the human umbilical vascular endothelial cells. Two compounds (**85** and **89**) showed good inhibitory ability to prevent sprouting and closed polygon formation of the tubular networks, promising them to be lead compounds. Compound **89** showed 60% inhibition at 0.051 M.

Most of the small molecule inhibitors of VEGFR kinase (Fig. **12**) are ATP competitive, by binding to the ATP-pocket of the kinase domain, among them:

(a) SU5416 [113], **93:** is a potent inhibitor of the VEGF receptor kinases and displayed encouraging anti-angiogenic effects and potent anti-tumor activity in a wide range of animal models and proceeded into clinical studies both as a single agent and in combination studies. However, a major drawback was that the compound was not orally bioavailable and poor solubility properties with unacceptable side-effects led to SU5416 being discontinued.

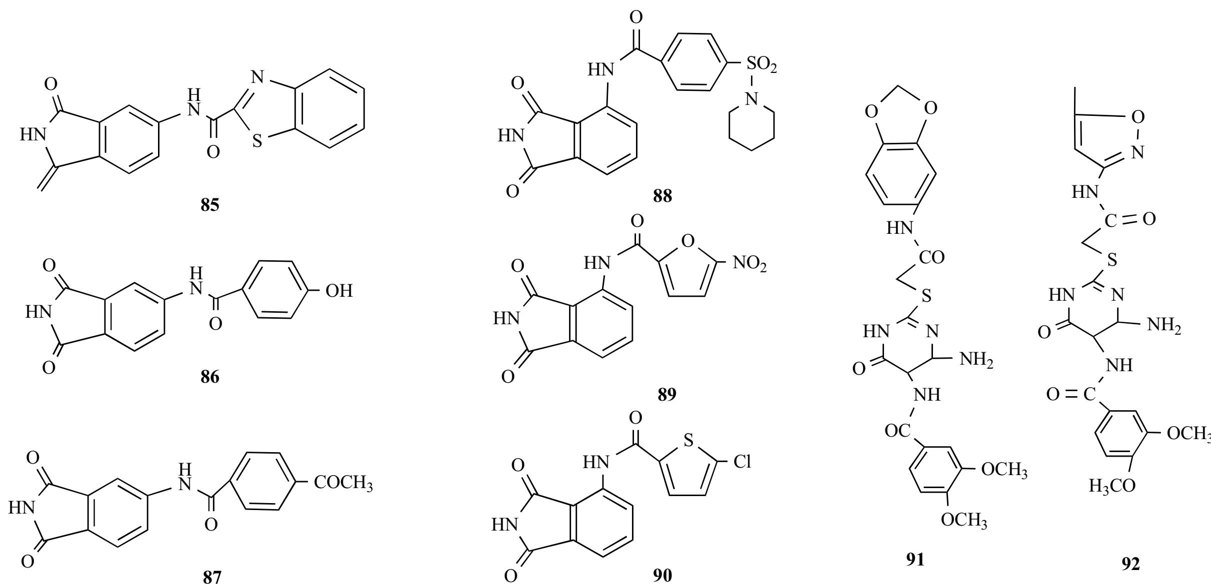


Fig. (11).

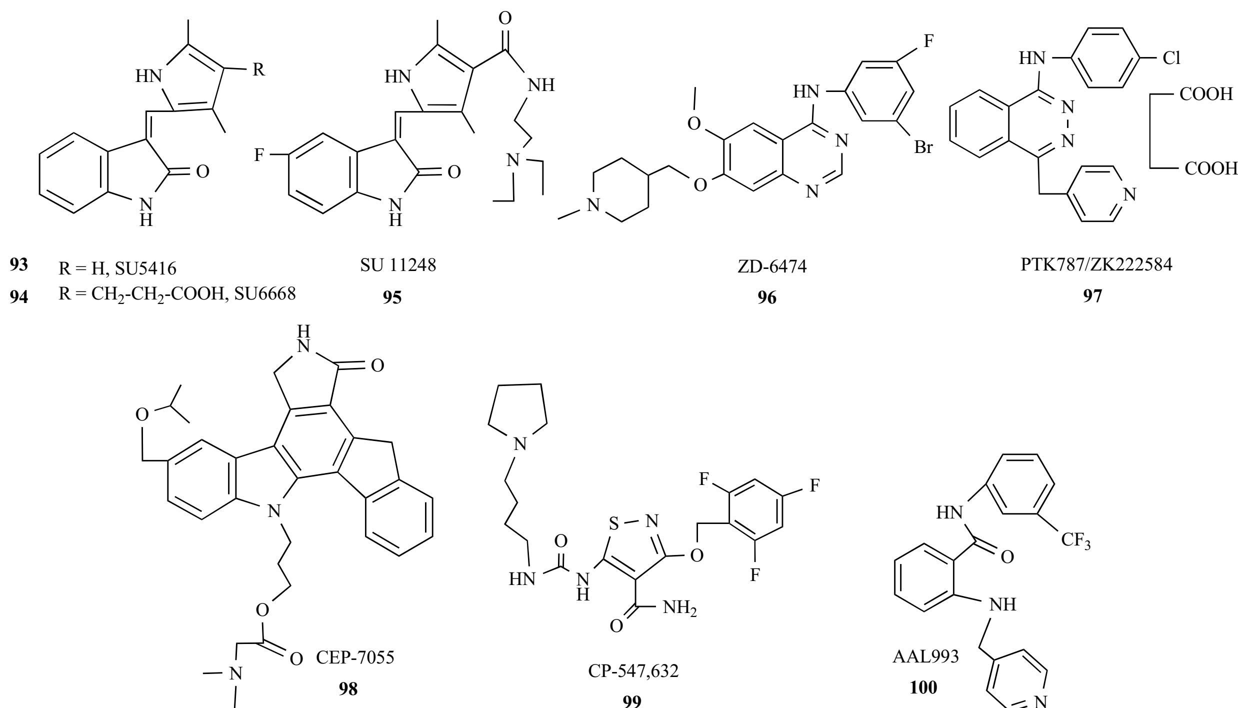


Fig. (12).

Table 10. Clinical Studies with Small Molecule VEGF-R Kinase Inhibitors IC50 (nM)

VEGFR Kinases	SU5416	SU6668	SU 11248	ZD-6474	PTK787/ZK222584	AAL993
VEGF-R1	43 ± 11	15 ± 3	15 ± 183 ± 9	110 ± 27	130 ± 81	
VEGF-R2	220 ± 34	200 ± 15	38 ± 11	17 ± 3	42 ± 3	23 ± 6
VEGF-R3	54 ± 4	10 ± 2	30 ± 6	233 ± 52	195 ± 30	18 ± 1
PDGFR-h	68 ± 2	39 ± 1	55 ± 1	477 ± 84	490 ± 52	--------
CSF-1R	84 ± 4	45 ± 2	35 ± 6	------	1240 ± 180	--------
Flt-3	35 ± 4	26 ± 7	21 ± 5	343 ± 76	-------	--------
c-Kit	660 ± 165	750 ± 120	211 ± 34	------	620 ± 56	--------
FGF-R1	------	------	675 ± 69	-----	-------	--------
InsR	------	------	560 ± 140	186 ± 19	--------	--------
c-Src	------	------	1000	------	--------	--------
CDK1	------	------	2600	------	-------	--------
c-Abl	------	------	610	86 ± 21	--------	--------

(b) SU6668 [113], **94**: The much more soluble acid analogue, SU6668, shows a similar selectivity profile to that of SU5416, but having little effect on other kinases at concentrations below 5 AM.

(c) SU11248 [113], **95**: The third compound based upon the indolinone template, SU11248, which incorporates a tertiary amine moiety to confer good solubility properties. SU11248 inhibits a wide range of protein kinases, although it does display selectivity towards the PDGFR family of transmembrane receptor tyrosine kinases.

(d) ZD6474 [113], **96**: Another first-generation compound is ZD6474 which resembles the ErbB-1 kinase inhibitor in being structurally based upon a quinazoline template. The compound possesses good biopharmaceutical properties, resulting in an excellent pharmacokinetic profile and, as a result of VEGF and EGF inhibition, efficacy as an anti-

angiogenic and antitumor agent following oral administration to animals. Notwithstanding, ZD6474 has an acceptable safety profile for cancer therapy and the compound is continuing into phase III clinical studies.

(e) PTK787/ZK222584 [114,115], **97**: Of the first-generation VEGF-R kinases inhibitors, the phthalazine derivative vatalanib (PTK787/ZK222584) is the most selective. This compound possesses good oral bioavailability and has demonstrated anti-angiogenic and anti-tumor activity in a range of animal models.

(f) AAL993 [116], **100:** Based upon theATP-binding conformation of the kinase model and the key pharmacophore elements of PTK787, it was postulated that an anthranilamide scaffold in which an intramolecular H-bond between the aniline-NH and the benzanilide-CMO would favour a conformation having a high degree of similarity to that of PTK787. Lead optimization within the anthranilamide series resulted in the identification of AAL993 as a highly potent and selective inhibitor that possesses good biopharmaceutical properties and displays excellent oral bioavailability. Table **1** compiles clinical studies with small molecule VEGF-R kinase inhibitors IC_{50} (nM) and further new generations of anti-VEGFR compounds based on a variety of chemical scaffolds are now emerging, as exemplified by AAL993, CEP-7055, **98** [117], and CP-547632, **99** [118].

Protein tyrosine kinases are essential for the activation and proliferation of inflammatory cells and airway-resident cells. Protein tyrosine kinases can be divided into two families: receptor tyrosine kinases and non-receptor tyrosine kinases [119]. The family of receptor tyrosine kinases includes receptors for various growth factors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), nerve growth factor (NGF) and basic fibroblast growth factor (bFGF). These receptors are activated by ligand-induced receptor dimerization and tyrosine autophosphorylation, followed by recruitment and activation of signaling molecules that contain src homology 2 (SH2), SH3, phosphotyrosine binding (PTB) and/or Pleckstrin homology (PH) tandem domains, which include the non-receptor tyrosine kinase Src, protein tyrosine phosphatase SHP2, protein adapter Shc, Ras-GAP, phospholipase Cg (PLCg) and phosphatidylinositol 3-OH kinase (PI3K), resulting in functional responses [120].

The family of non-receptor tyrosine kinases can be divided into eleven subfamilies, including Src, Syk, Janus tyrosine kinase (JAK), Tec, and Csk, among many others [121]. They have been shown to initiate multiple signaling pathways resulting in activation of mitogen-activated protein kinase (MAPK), PI3K, signal transducer and activator of transcription (STAT) and nuclear factor-nB (NF-nB), and a host of inflammatory cell functions including degranulation, proliferation, differentiation and apoptosis in response to antigens, cytokines, chemokines, and various pro-inflammatory mediators. In the literature there are two types of tyrosine kinase inhibitors are known: a) non-selective inhibitors and b) selective inhibitors. As compiled in Fig. **13**, non-selective tyrosine kinase inhibitors include Genistein **101** [122-124] [4',5,7-trihydroxyisoflavone], Tyrphostin AG213 **102** [123,125] [α-cyano-(3,4-dihydroxy) thiocinnamamide; RG50864], Tyrphostin AG126 **103** [126] [α-cyano-(3-hydroxy-4-nitro)cinnamonitrile], ST638 **104** [127] [α-cyano-(3-ethoxy-4-hydroxy-5-phenylthiomethyl)cinnamide], Lavendustin A **105** [122,128] [5-amino-[(N-2,5-dihydroxybenzyl)-N'-2-hydroxybenzyl]salicylic acid; RG14355], XR774 **106** [129] [(6bS,7R,8S)-7-methoxy-4,8,9-trihydroxy-1,6b,7,8 tetrahydro-2H-benzo[j] fluoranthen-3-one]. Selective tyrosine kinase inhibitors including LFM-A13 **107** [α-cyano-b-hydroxy-b-methyl-N-(2,5-dibromophenyl)propenamide] [130], PP1 **108** [4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d-pyrimidine] [131,132], PP2 **109** [4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine], SU6656 **110** [2-oxo-3-(4,5,6,7-tetrahydro-1H-indol-2-ylmethylene)-2,3-dihydro-1*H*-indole-5-sulfonicacid dimethylamide] [133], Nocodazole **111** [methyl-[5-(2-thienylcarbonyl)-1*H*-benzimidazol-2yl]-carbamate,R17934] [134], Damnacanthal **112** [3-hydroxy-1-methoxy-anthraquinone-2-aldehyde] [135] Tyrphostin AG490 **113** [α-cyano-(3,4-dihydroxy)-N-benzylcinnamamide] [136,137], WHI-P131 **114** [4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinonazoline] [138], WHI-P97 **115** [4-(3',5'-dibromo-4'-hydroxyphenyl)amino-6,7-dimethoxyquinazoline] [139], Piceatannol **116** [3,4,3',5'-tetrahydroxy-*trans*-stilbene] [140,141], Tyrphostin AG1478 **117** [4-(3-chloroanilino)-6,7-dimethoxyquinazoline] [142].

The epidermal growth factor (EGF) is the prototype of a large family of peptide ligands that bind to cell membrane receptors and activate a myriad of intracellular signaling pathways to control tumor cell growth, proliferation, survival, metastasis, and angiogenesis [143]. A number of small molecule inhibitors of EGF receptor (EGFR) tyrosine kinase are known including the anilinoquinazoline derivatives ZD 1839, OSI-774, quinazoline GW572016, pyrrolopyrimidine PKI-166, CI-1033 (PD183805) and EKB-569.8 ZD1839 has shown excellent activity *in vitro* and in pre-clinical studies against a variety of tumor cells when used alone or in combination with other chemotherapeutic agents [144–146]. In the recent work reported by Cavasotto *et al.* [147] the crystal structure of EGFR has been used for the first time to perform a virtual screening of a large chemical library and select only those compounds that best fit into the binding site for subsequent biological evaluation. Figure **14** shows compounds that exhibited more than 40% inhibition of EGFR tyrosine kinase activity. The discovery of N-(1,3-benzodioxol-5-ylmethyl)-1-(3-methylphenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**120**) as a low-micromolar EGFR inhibitor with a unique binding mode opens a new avenue toward the development of novel drug scaffolds as potent and selective inhibitors of EGFR tyrosine kinase activity. Among the different EGFR inhibitors described here, **120** is not the one with highest growth inhibitory activity against cancer cells. Other molecules (**118** and **119**) elicited stronger antiproliferative activity even at concentrations at which no inhibition of EGFR kinase activity was observed *in vitro*.

Zhang *et al.* [148] reported the identification of heparin-growth factors interactions from chemical libraries based on Ugi four-component condensation reactions of isocyanides. The functional groups contained in the four building blocks were sulfonic acid, carboxylic acid, hydroxyl groups, as these mimic groups are present in the native heparin structure. Building blocks of carboxylic acids, primary amines

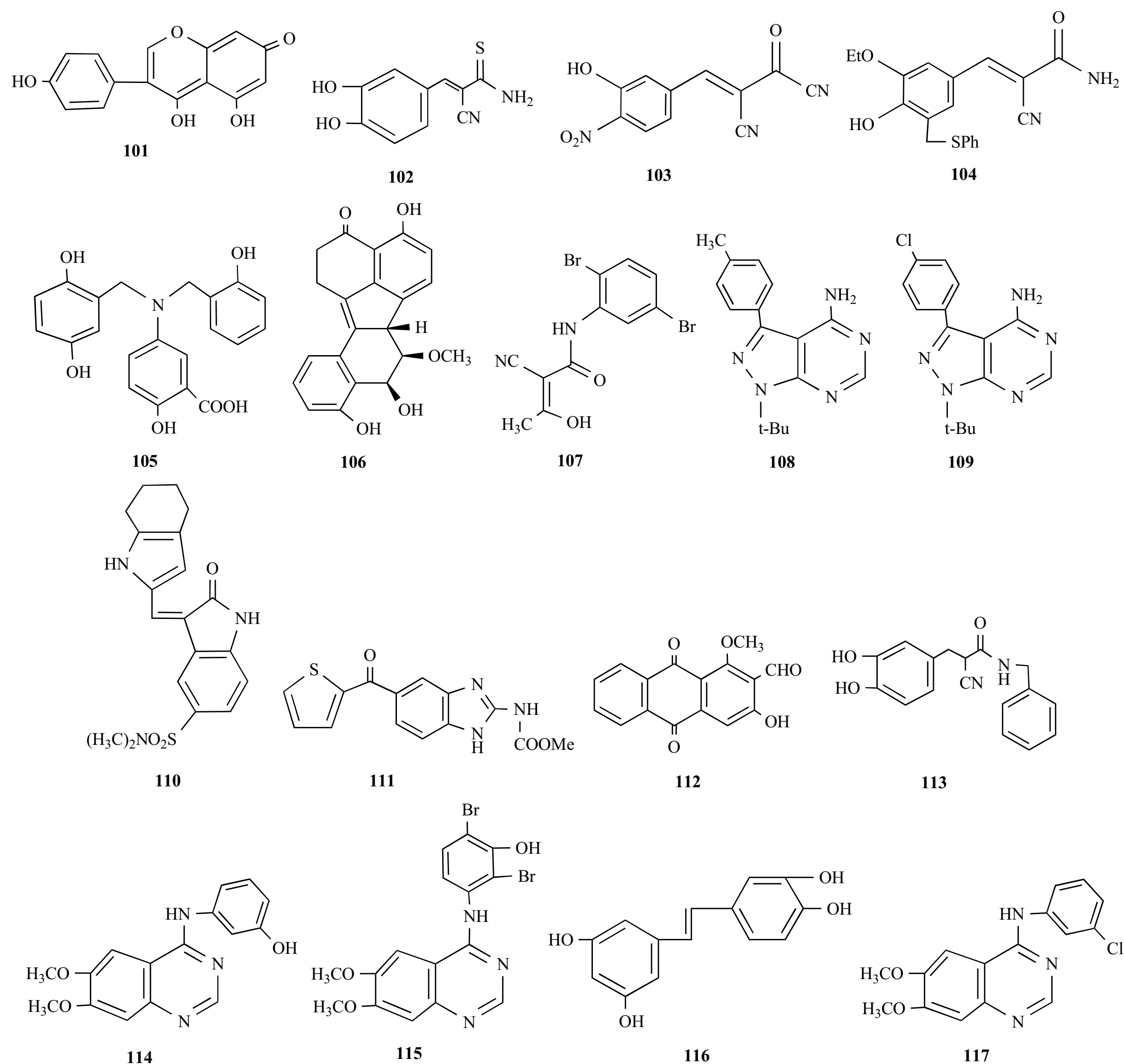


Fig. (13).

and aldehydes that contain unprotected hydroxyl groups were used directly in the condensation while the hydroxyl groups of isocyanides were protected as esters. Parallel solution-phase combination of these building blocks yielded a sum of 9600 small molecules type (**125**-**126**) for screening (Scheme **3**). Reactions of one of the isocyanides used with 1,6-diisocyanohexane provided extended structures of symmetric dimmers (**127**-**138**). Structural studies have shown that these types have the best potential for interaction with the growth-factor binding site. To increase the number and diversity of extended structure, adipic acid and 1,6-hexanediamine were used in the synthesis to furnish small molecule of the type (**139**-**143**). Biological results of the active compounds identified in the library are listed in Table **11**. Most of the active compounds showed moderate selectivity towards VEGF versus bFGF presumably due to the differences in the structure of VEGF and bFGF heparin-binding sites. These results provide direction to obtain a more specific structure-activity relationship (SAR) profile of this type of compounds and to obtain more potent and selective inhibitors.

Molecules based on derivatives of 2-(3-nitrobenzoyl) benzoic acid (KI) showed antagonistic effects against invasion and migration of human fibrosarcoma HT1080 cells as reported by Ishida *et al.* [149]. The compounds were synthesized using 2-(4-fluorobenzoyl)-benzoic acid **144** (KI-101) as a starting material (Scheme **5**). 2-(4-Fluoro-3-nitrobenzoyl)benzoic acid **145** (KI-102) was obtained by the nitration of **144**. KI-102 was treated with certain thiols, alcohols, and amines to give **146** (KI-103) through **152** (KI-109). Screening results revealed that **148** (KI-105) inhibited both invasion and migration and can increase the adherence of HT1080

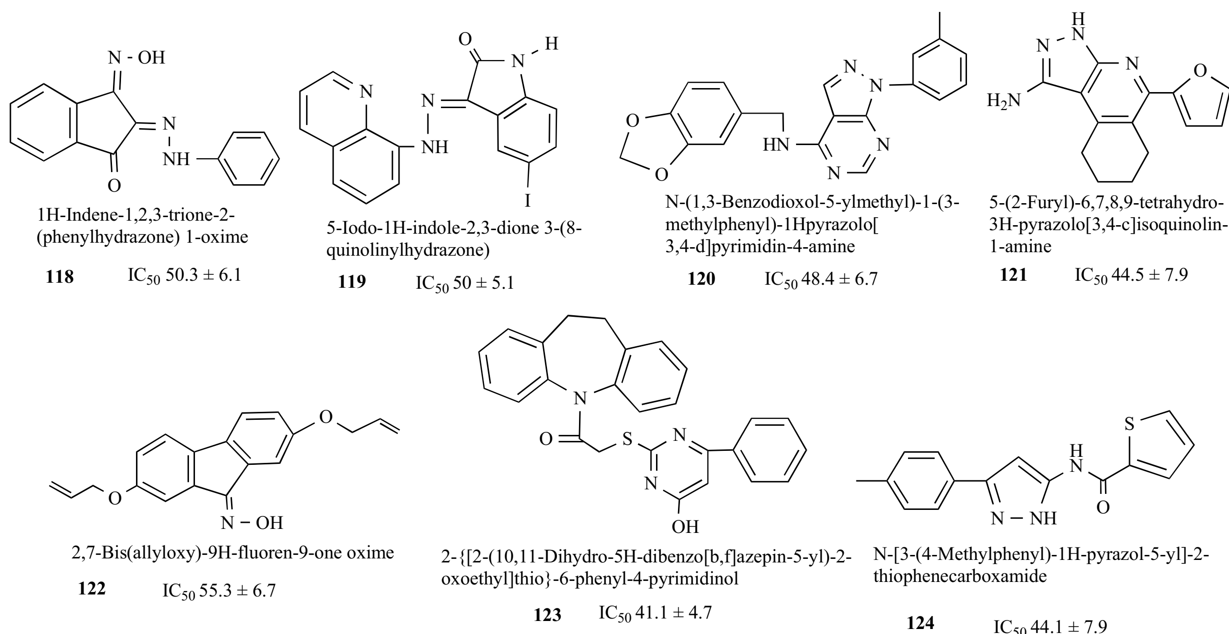


Fig. (14).

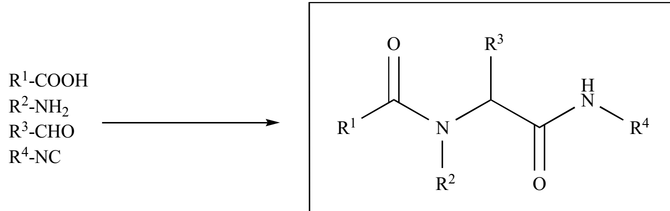
cells. The inhibition percentages of migration and invasion by **148** at 100 µM were 62% and 69%, respectively. Heparan sulfate (HS) inhibited only invasion, and the inhibition at 100 µM of HS in disaccharide unit was 78%.KI-105 also showed moderate inhibition of heparanase (IC_{50} 300 mM). Heparanase has been considered a major enzyme that degrades HSGAGs in mammalian tissues [150] and human tumors [151]. Heparanase inhibition by KI-105 may correlate with an increase in the amount of cell-surface HSGAGs on HT1080 cells. Therefore, it is conceivable that the increase in cell-surface HSGAGs by KI-105 treatment was caused not by the enhancement of biosynthesis of HSGAGs but the inhibition of degradation of cell-surface HSGAGs by heparanase.

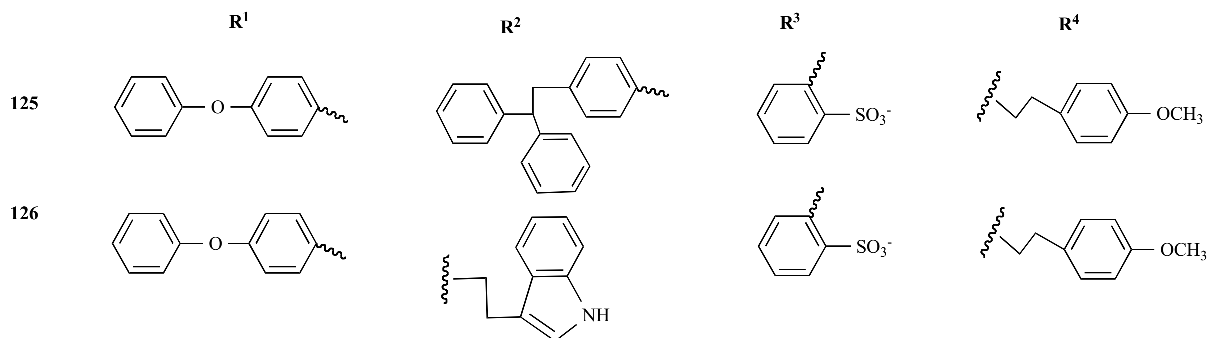
2.6. High Molecular Weight Cationic Polymers

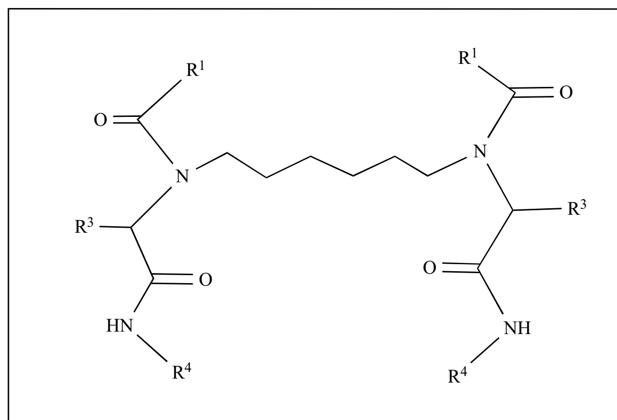
Different types of high molecular weight polymers such as sulfonated polymers, polyaromatic anionic compounds, polymers with amino acid side chains, polymers with mono- and disaccharide side chains are known as synthetic mimics of the biological properties of heparin.

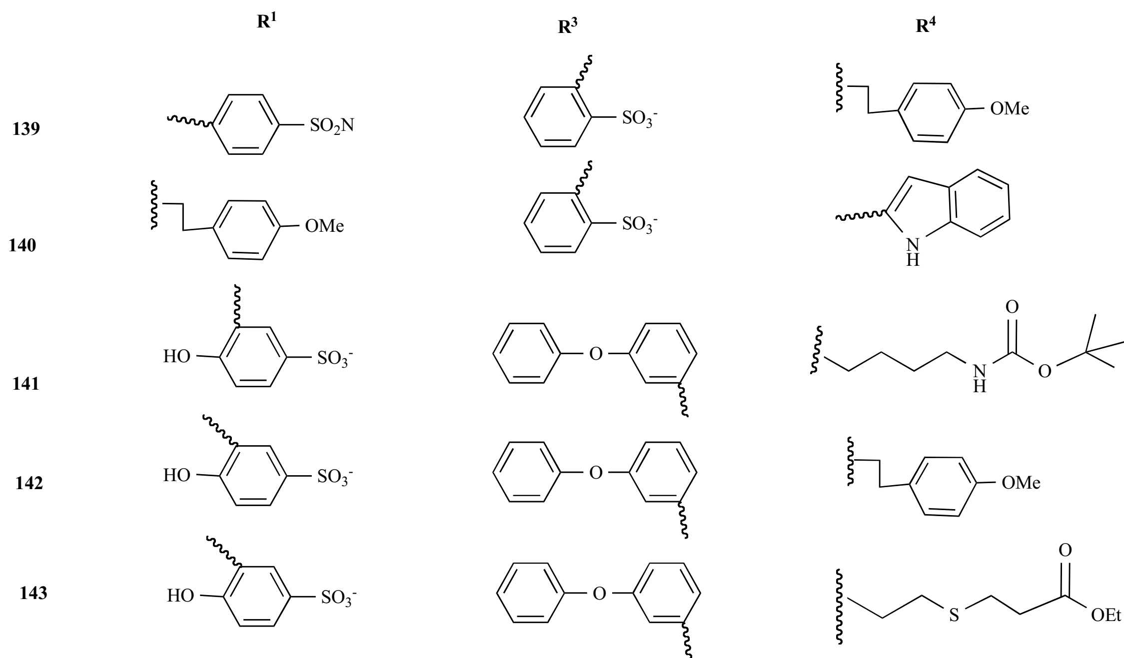
Poly (β-amino esters) are one of a number of types of basic polymers that are used for delivering DNA into cells [152]. In pursuing this line of research, Berry and coworkers [153] found that heparin could block cellular uptake of the poly (β-amino ester)–DNA complex. Moreover, these poly (β-amino esters) promoted cellular uptake of heparin, particularly highly sulfated full-length (molecular weight average 12,000) heparin, suggesting that heparin was competing with DNA for poly (β-amino esters). A small library of varied poly (β-amino esters) was next examined for the members abilities to bind and internalize heparin. Of the70 poly (β-amino esters) examined, most bound heparin but only **153**-**157** enabled their internalization (Fig. **16**). This selectivity suggests that an appropriate match between the structure of heparin and the structure of the basic polymer is essential in promoting cellular uptake. Furthermore, the localization of intracellular heparin was not limited to endosomes and lysosomes. One of the most interference with the fibroblast growth factor signal transduction pathway, was shown to be unaffected by internalized heparin. Detailed studies by Berry and others [153] suggest that poly (β-amino ester)–heparin complexes affect cellular processes including inducing transcription factor and caspase activation, ultimately inducing apoptotic cell death. The heparin-poly (β-amino ester) complex appears to be internalized through endocytosis and may enter the cytosol through lysosomal escape mediated by cationic poly (β-amino ester), Fig. **17**, [154].

Polysulfonated compounds with distinct chemical structures and sizes act as FGF-2 antagonists by mimicking some of the functional features of soluble heparin/HS. This is shown by the capacity of polysulfonates to impair the binding of FGF-2 to both HSPGs and FGFRs in cultured endothelial cells. Likewise, PSS [poly (4-styrenesulfonic acid)] **158**, PAS [poly (anetholesulfonic acid)] **159**, PVS [poly (vinylsulfonic acid)] **160**, and PAMPS [poly (2-acrylamido-2-methyl-1-propanesulfonic acid)] **161**, (Fig. **18**) were found to abrogate FGF-2-mediated attachment of HSPG-deficient FGFR-1-transfected CHO mutants to a monolayer of wild-type HSPG-bearing CHO-K1 cells, which indicates that polysulfonates prevent the formation of the HSPG/FGF-2/FGFR ternary complex [155]. As reported by Liekens *et al.* [156], direct interaction of the polysulfonates with FGF-2 was demonstrated by their ability to protect the growth factor from proteolytic cleavage. Accordingly, molecular modeling, based on the crystal structure of the interaction of FGF-2 with a heparin hexamer, showed the feasibility of docking PAMPS into the heparin binding domain of FGF-2. In agreement with their FGF-2-binding capacity, PSS, PAS, and PAMPS inhibited FGF-2-induced cell proliferation in


R1-COOH
R2-NH2
R3-CHO
R4-NC
O
R3
H
N
R1
N
R4
R2
O


R1
R2
R3
R4
125
O
SO3-
OCH3
126
O
NH
SO3-
OCH3


R1
R1
O
O
N
N
R3
R3
O
O
HN
NH
R4
R4


R1
R3
R4
139
SO2N
SO3-
OMe
140
OMe
SO3-
N
H
141
HO
SO3-
O
O
N
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142
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OMe
143
HO
SO3-
O
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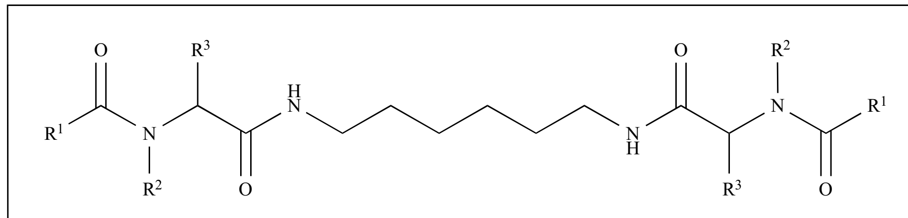
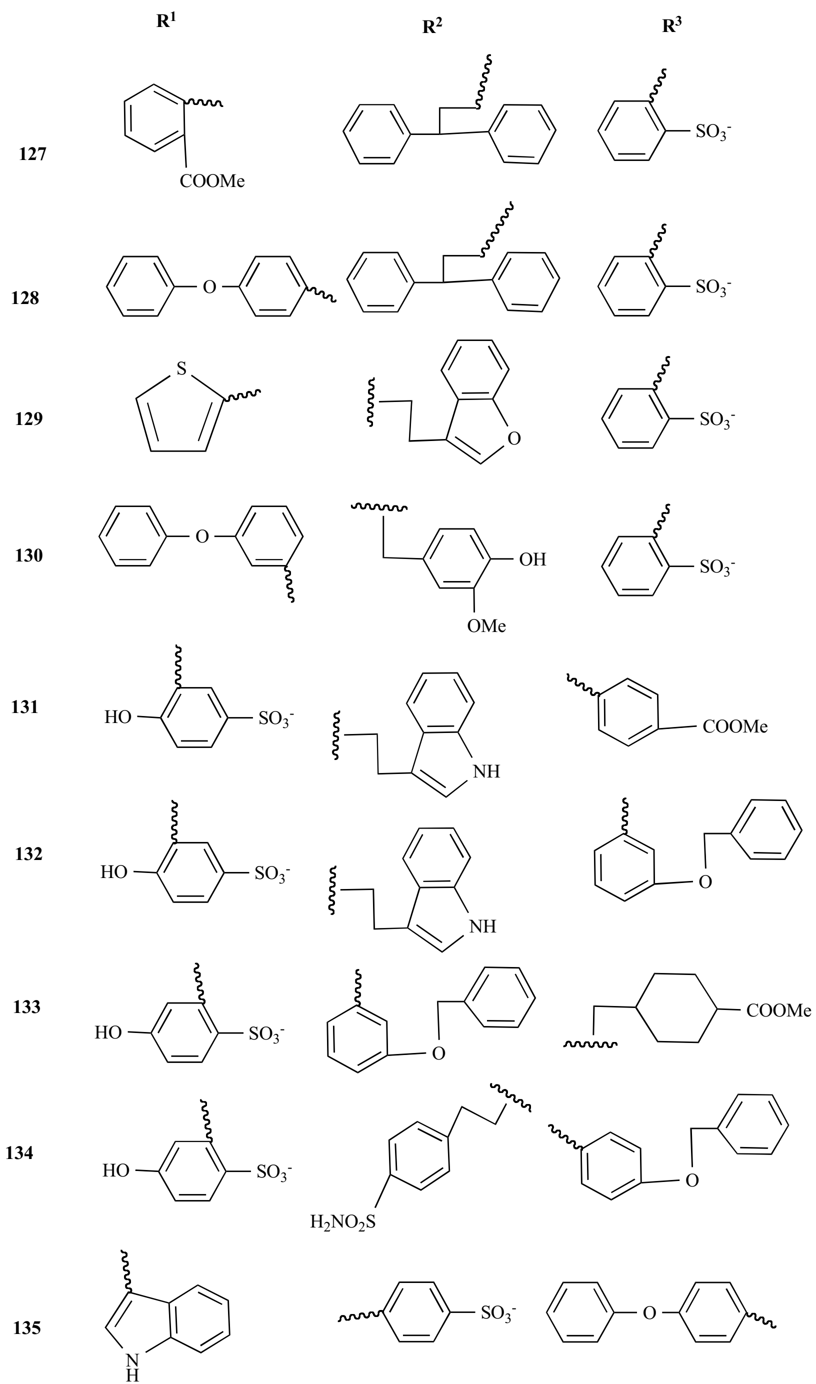
S
OEt

(Fig. 15. Contd….)





(Fig. 15. Contd….)

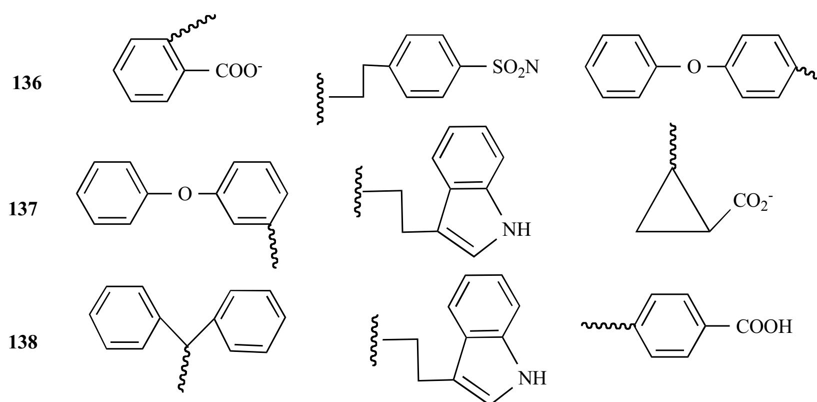


Fig. (15).

GM 7373 cells and murine brain microvascular endothelial cells. The antiproliferative activity of these compounds was associated with the abrogation of FGF-2- induced tyrosine phosphorylation of FGFR-1. Moreover, the polysulfonates PSS and PAS inhibited FGF-2-induced activation of mitogen-activated protein kinase-1/2, involved in FGF-2 signal transduction. These compounds may provide a tool to inhibit FGF-2-induced endothelial cell proliferation in angiogenesis and tumor growth.

Table 11. Biological Screening of Inhibitors of Heparin Binding to VEGF and bFGF

Cpd	IC50 μM VEGF	IC50 μM bEGF
125	5.5± 0.5	35.5± 2.5
126	13.5± 2.5	>50
127	7.95± 0.6	45± 3.2
128	1.65± 0.15	10.2± 2.8
129	5.3± 0.20	27.5± 2.5
130	14.5± 0.5	>50
131	4.8± 0.00	>50
132	5.75± 0.45	25± 2.0
133	5.75± 0.75	16.5± 7.5
134	6.0± 1.00	36.00
135	2.1± 0.6	5.2± 1.2
136	2.25± 0.4	25.5± 1.8
137	3.2± 0.6	21.5± 0.5
138	4.7± 0.1	18.5± 1.5
139	11.5± 1.5	>50
140	5.7± 0.6	42.0± 8.0
141	6.7± 1.1	>50
142	9.5± 0.5	47.5± 2.5
143	9.9± 0.1	>50

Synthesis of polymers with pendent saccharides, also known as "glycopolymers" attracted the attention of many researchers in the past few years [157-160]. For example, vinyl-, acrylate-, or norbornene-containing saccharide derivatives can be polymerized to yield glycopolymers with biologically active hydrophilic saccharides in the side chains. Moreover, the backbone structure may be altered to modulate both polymer biostability and processability. Significantly, the carbohydrate density of the polymer chain, which appears to affect both ligand binding affinity, as well as polymer physiochemical properties, can be controlled through the choice of polymerizable saccharide, associated co-monomers, and dictated chain size. Overall, the potential to stably link bioactive oligosaccharide ligands is an important feature of the glycopolymer approach and presents an opportunity to create GAG mimicking structures that facilitate optimized sequestration and controlled local release of bioactive protein species.

Polyacrylamide- based neoglycoconjugates have been used as diagnostic reagents, such as in the inhibition of hemagglutination by pathogens [161] or as solid-phase coatings in enzyme-linked immunosorbent assays (ELISA) [162,163]. Chaikof and coauthors [164] have confirmed that cyanoxyl (OCN)-mediated free-radical polymerization of acryl-derivatized glyco-monomers is a convenient tool to produce water-soluble glycopolymers. As shown in Scheme **6**, cyanoxyl radicals were generated by an electron-transfer reaction between cyanate anions ($^{\bullet}OC\equiv N$), from a NaOCN aqueous solution, and *p*-chlorobenzene-diazonium salts ($ClC_6H_4N\equiv N$-BF_4^-) **163**, that were previously prepared *in situ* through a diazotization reaction of *p*-chloroaniline **162** in water. In addition to cyanoxyl persistent radicals, aryl-type active radicals were simultaneously produced, and only the latter species is capable of initiating chain growth.

Cyanoxyl-mediated homo-polymerization of sulfated *N*-acetylglucosamine glycomonomer **165** or lactose heptasulfate monomer **167** generated the expected sulfated glycopolymers **168, 169** in good conversion yield (60-80%). Polymers of varying molecular weight were prepared by altering either monomer conversion or the initial ratio of monomer to initiator concentrations. Similarly, copolymers were prepared

a) fuming HNO_3 4ºC, 2 hr; b) sodium ethanethioate, rt,4h; c) mercaptosuccinic acid, DIPEA; d) thiophenol, DIPEA; e)4-mercaptobenzoic acid, DIPEA; f)ethylamine/THF, DIPEA; g) cyclohexylamine, DIPEA; h) sodium ethoxide, THF.

Scheme 6.

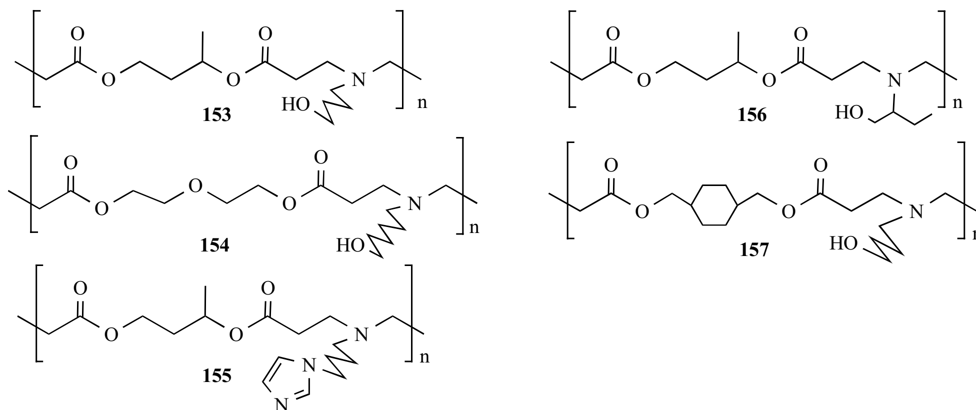


Fig. (16).

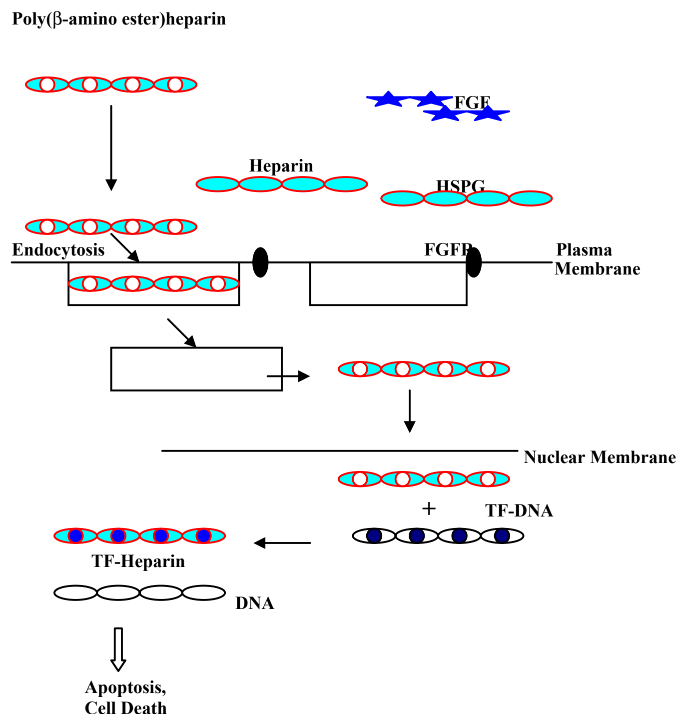


Fig. (17). Proposed endocytotic mechanism for heparin cellular uptake of heparin poly (β-amino ester) complex escape, distribution through the cell, displacement of DNA, complexation with transcription factor, and resulting apoptosis and cell death. Fibroblast growth factor (FGF) and fibroblast growth factor receptor (FGFR) interact with heparan sulfate proteoglycans (HSPG) and form a complex that can be similarly transported into the cell [154].

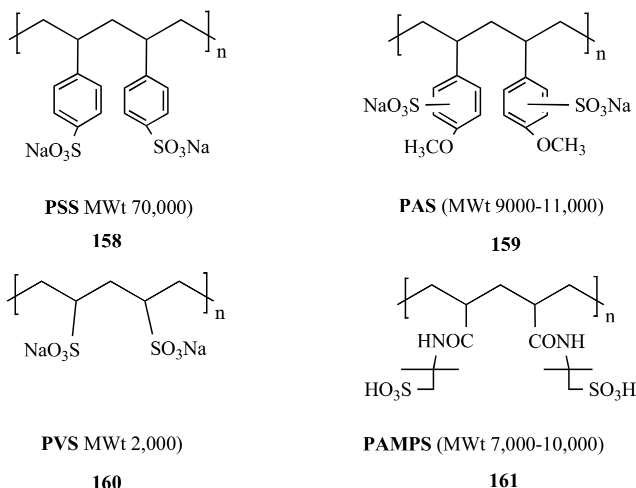
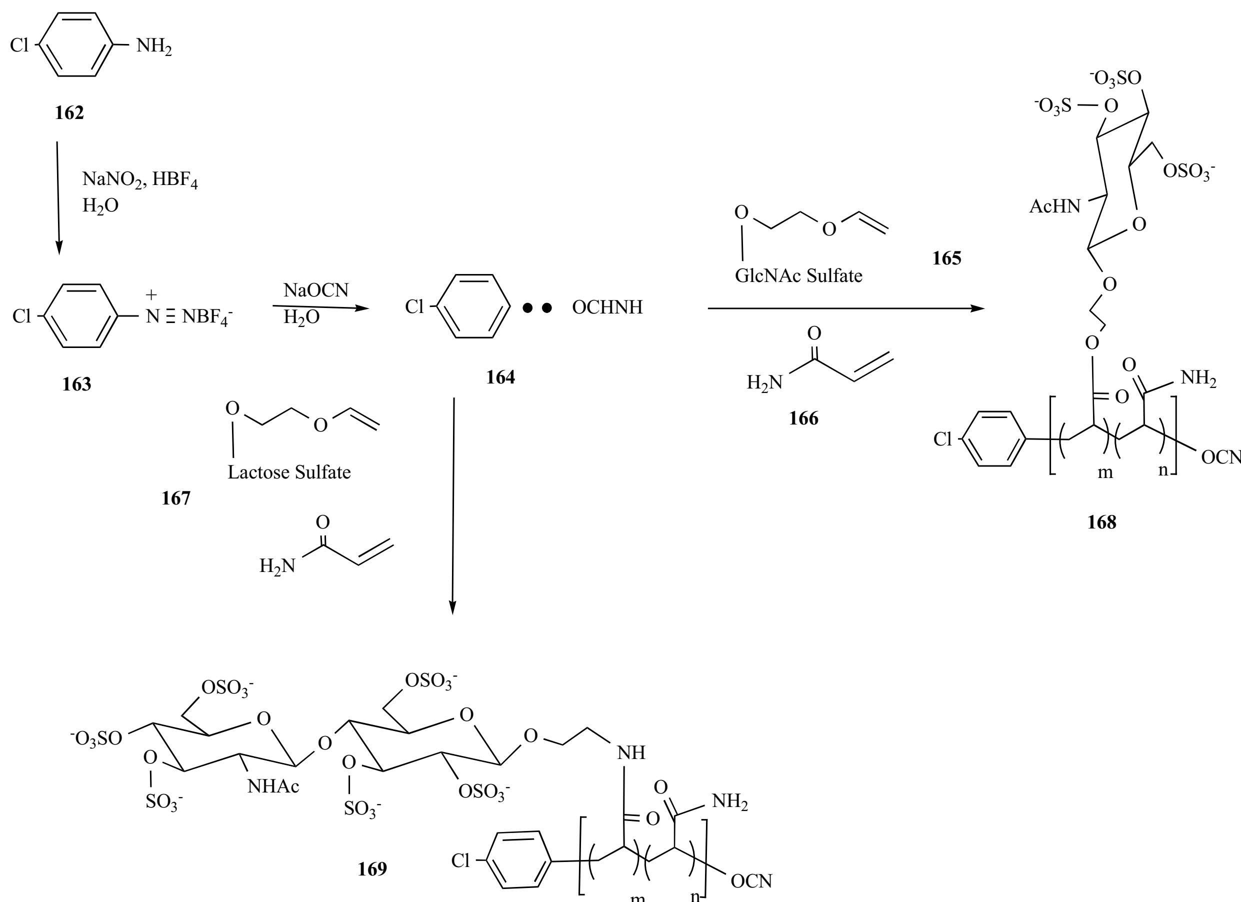


Table 12. Inhibition of the Mitogenic Activity ID_{50} (µM)

PSS	0.004
PAS	0.09
PVS	-------
PAMS	3.3

Fig. (18).

by copolymerization of sulfated glycomonomers with acrylamide. Biological results demonstrated that a glycopolymer bearing pendant, fully sulfated lactose units effectively replaces heparin and heparan sulfate as a molecular chaperone for FGF-2. Specifically, a compound was identified that protects FGF-2 from proteolytic, acid, and heat-induced degradation, while selectively promoting growth factor and receptor dimerization. Significantly, the capacity of this heparin-mimic to promote an FGF-2 specific proliferative cell response was confirmed and suggests potential applications for this compound and related derivatives in areas related to therapeutic angiogenesis. It is worthy to note that this polymerization technique can be conducted in aqueous solution, is tolerant of a broad range of functional groups (OH, NH_2, COOH, and OSO_3-), yields low-polydispersity polymers (PDI < 1.50) with high saccharide content, and can be applied to the synthesis of block and graft copolymers.



Scheme 7.

Acid-catalyzed polymerization of phenols and formaldehyde was used to synthesize negatively charged, nonsulfated polyanionic compounds [165]. Aromatic ring monomers were chosen and strong oxidizing reagents avoided so that the polymerization process with formaldehyde would yield substantially ordered and defined backbones. Using this approach, a series of polyanionic polymers was identified with repeating phenol-based monomers, including poly-4-hydroxyphenoxy acetic acid, RG-13577, that mimics various effects of heparin. Some of these nontoxic, nonsulfated linear polyanionic compounds were found to revert the transformed phenotype of FGF-2 transfected cells [166] and inhibit the proliferation of vascular smooth muscle cells induced by thrombin, FGF-2, and serum [167]. Miao *et al.* [168] reported that a synthetic, nonsulfated polyanionic aromatic compound (RG-13577) that mimics functional features of heparin/HS. FGF-2–stimulated proliferation of vascular endothelial cells was markedly inhibited in the presence of 5–10 μg/ml compound RG-13577 (poly-4-hydroxyphenoxy acetic acid; Mr ~ 5 kD). Direct interaction between RG-13577 and FGF-2 was demonstrated by the ability of the former to compete with heparin on binding to FGF-2. RG-13577 inhibited FGF-2 binding to soluble- and cell surface–FGF receptor 1 (FGFR1). Unlike heparin, RG-13577 alone failed to mediate dimerization of FGF-2. Moreover, it abrogated heparin-mediated dimerization of FGF-2 and FGFR1, as well as FGF-2 mitogenic activity in HS deficient F32 lymphoid cells. The antiproliferative effect of compound RG-13577 was associated with abrogation of FGF 2–induced tyrosine phosphorylation of FGFR1 and of cytoplasmic proteins involved in FGF-2 signal transduction, such as p90 and mitogen-activated protein kinase. A more effective inhibition of tyrosine phosphorylation was obtained after removal of the cell surface HS by heparinase. In contrast, tyrosine phosphorylation of an ~ 200-kD protein was stimulated by RG-13577, but not by heparin or FGF-2. RG-13577 prevented micro-vessel outgrowth from rat aortic rings embedded in a collagen gel. Development of nontoxic polyanionic compounds may provide an effective strategy to inhibit FGF-2 induced cell proliferation associated with angiogenesis, arteriosclerosis, and restenosis.

CONCLUSION

Molecules that mimic the sulfated glycosaminoglycan heparin and bind to heparin-binding growth factors would serve as important building blocks for synthetic biomaterials. Different types of synthetic mimics of the biological properties of heparin have been prepared including high molecular weight compounds such as sulfonated polymers, polyaromatic anionic compounds, polymers with amino acid side

chains, polymers with mono- and disaccharide side chains or small molecule mimics include β-cyclodextrin tetradecasulfate, suramin and its analogs, and molecules based on derivatives of 2-(3-nitrobenzoyl)benzoic acid and acylamino acid amides. Among such compounds are also pentosan polysulfate, laminarin sulfate, sucrose octasulfate, and aurin tricarboxylic acid (ATA). Peptide-based mimics of heparin functionality have been limited. Compared to heparin and many mimics, the degree of sulfation in the peptides is low and thus they are natural targets as heparin mimics. Peptides can be coupled with the relative ease to biomaterials and may exhibit lower toxicities compared to compounds such as suramin. Aromatic sulfonamide derivatives exhibit a range of bioactivities such as antimicrobial, antidiabetic, anti-inflammatory, and anticancer. A novel angiogenesis inhibitor E 7820; *N*-(3-Cyano-4-methyl-1H-indol-7-yl)-3-cyanobenzene-sulfonamide among other sulfonamides was discovered and it is used as a TF model for screening assay.

The anticoagulant activity of the known heparin pentasaccharide sequence prompted synthetic efforts aimed at the procurement of this structure as well as a host of related sequences. The synthetic heparin oligosaccharide and its α-methyl-glycoside analog are displaying the same biological properties. Replacement of the reducing end glucosamine by a glucose residue indicated that *O*-sulfates are effective substitutes for *N*-sulfates. Introduction of an extra 3-*O*-sulfate group at the reducing end of pentasaccharide increased factor activation of AT III Xa affinity. Spaced fully sulfated disaccharides attached by various linkers to the aromatic spacer have a remarkable antiproliferative activity significantly higher than heparin, which points at the contribution of the spacer to the overall binding.

A variety of non-peptide non-saccharides inhibitors as anti-angiogenesis therapies directed against the VEGFR kinase have been a promising and well-validated therapeutic approach under active evaluation of their safety and efficacy in multiple clinical trials.

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