Arylamidonaphtalene Sulfonate Compounds as a Novel Class of Heparanase Inhibitors

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Abstract. The search for antimetastatic agents for cancer therapy may involve the ability of new compounds to maintain the tissue extracellular matrix integrity. Among known factors, heparanase, an endoglucuronidase responsible for heparan sulfate cleavage, is a promising target whose inhibition could represent a strong obstacle for metastatic cancerous mechanisms. The antimetastatic activity of some suramin derivatives reported in literature suggests a possible involvement of the heparanase enzyme. To confirm such hypothesis, we have investigated FCE27266, a molecule known for its antiangiogenic and antimetastatic properties. Other new derivatives were also synthesized and investigated. Our findings revealed that FCE27266 as well as some derivatives have a strong heparanase inhibition activity, together with no cytotoxic power. Moreover, a FCE27266 analogue (SST0546NA1; **17a**) resulted also positive to lower gene expression of some proangiogenic factors.

Keywords: Antimetastatic activity; Heparanase inhibitors; Antitumor agents.

Heparan sulfate proteoglycans (HSPGs) are ubiquitous macromolecules associated with the cell surface and extracellular matrix (ECM) of a wide range of cells of vertebrate and invertebrate tissues.[1-3] The basic HSPG structure consists of a protein core to which several linear heparan sulfate (HS) glycosaminoglycan side chains are covalently O-linked.

HS glycosaminoglycans bind to and assemble a multitude of ECM proteins (i.e.,

laminin, fibronectin, collagen type IV) and thereby contribute significantly to the ECM self-assembly and integrity. Moreover, the HS chains ensure that a wide variety of bioactive molecules bind to the cell surface and ECM and thereby function in the control of normal and pathological processes, among which are morphogenesis, tissue repair, inflammation, vascularization and cancer metastasis.

Heparanase, an endoglucuronidase responsible for heparan sulfate (HS) cleavage, regulates the structure and function of heparan sulfate proteoglycans, thus resulting in structural alterations of the ECM and release of bioactive saccharide fragments and HS-bound growth factors and cytokines. There is growing evidence that heparanase upregulates expression of genes that participate in cancer metastasis and angiogenesis, glucose metabolism, immune response, inflammation and atherosclerosis, suggesting that heparanase belongs to an emerging class of proteins that play a significant role in regulating transcription in addition to their well-recognized extra-nuclear functions.[4-10]

A variety of compounds has been employed as inhibitors of this enzyme, both experimentally and in clinical trials. Literature describes mainly three classes of heparanase inhibitors, including active analogues of endogenous substance, synthetic small molecule compounds and natural products.[11-13]

As one of the closest mimics of HS, heparin is a natural choice as a heparanase inhibitor. However, clinical application as an anticancer/antimetastatic drug is limited due to its potent anticoagulant activity.[14] Considerable efforts have thus been expended in the development of modified heparins and related polysulfated compounds with reduced anticoagulant activity. In particular, modified heparins or sulphated oligosaccharides, such as PI-88 (Muparfostat), PG545, SST0001 (Roneparstat) and M-402 (Necuparanib) (Figure 1), have entered the clinical trials as potent heparanase inhibitors.

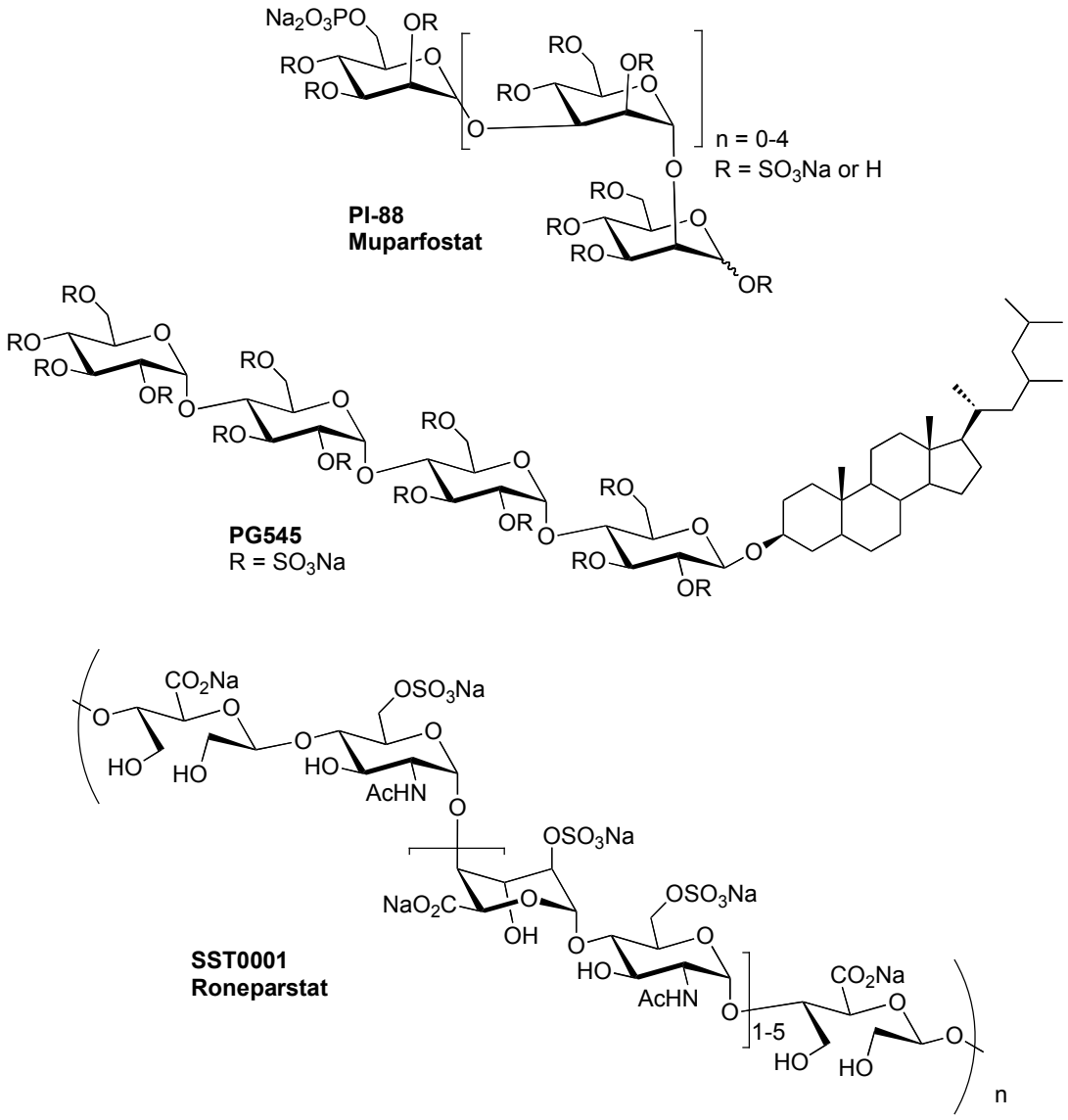


Figure 1. Modified heparins or sulphated oligosaccharides entered in clinical trials.

Among small molecules, suramin (Figure 2) a polysulfonated naphthylurea with antiangiogenic and antiproliferative activity, was found to inhibit heparanase with a *Ki* = 48 μM.[15] Suramin has undergone extensive clinical evaluation as inhibitor of growth factors bFGF, TNFα, VEGF and IL-8 as well as anticancer agent. However, suramin has found so far a limited clinical usefulness because of severe toxicity associated with indiscriminate inhibition of many other enzymes. Some suramin analogues, chosen because possessing higher antiangiogenic activity than suramin itself, were also described as heparanase inhibitors (IC_{50} = 20-30 μM).[16]

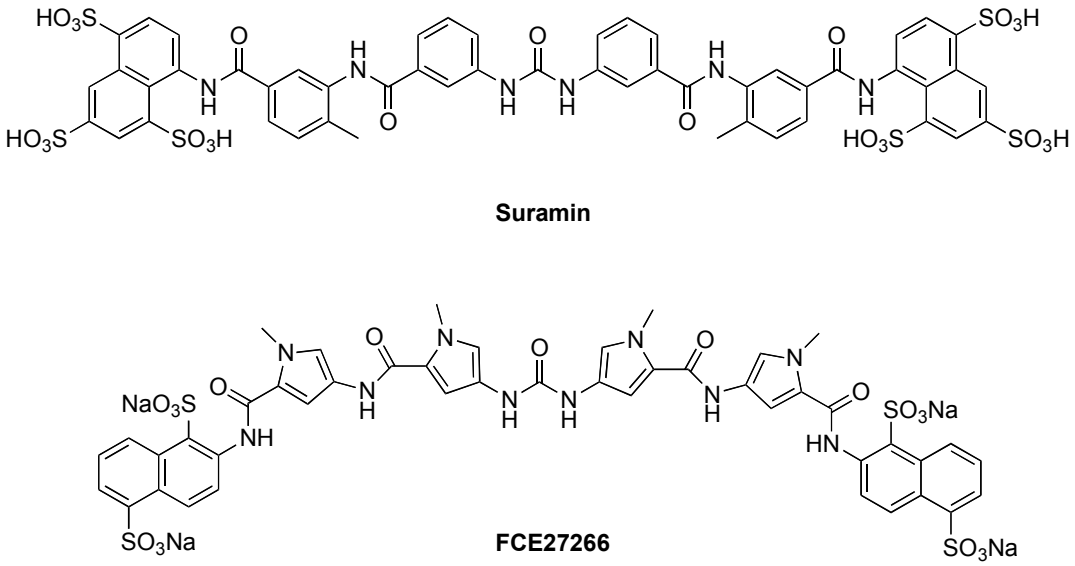


Figure 2. Polysulfonated naphthylurea derivatives with antiangiogenic and antiproliferative activity.

Besides studies on suramin, related sulfonated distamycin-A derivatives, sometimes reported as Suradistas, were prepared and studied.[17-21] In this series, compounds as **FCE27266**[18] (Figure 2) demonstrated the same promising pattern of antimetastatic and antiangiogenic action as suramin, but less cytotoxicity. Indeed, **FCE27266** has been reported as hybrid structure between Distamycin and Suramin, obtained by replacement of Suramin benzene moieties with N-methylpyrrole rings characteristic of Distamycins. The antimetastatic and antiangiogenic activities of **FCE27266** have been related to its ability to complex and inhibit several growth factors (bFGF, PDGFb, VEGF) as well as proteinases enzymes of the extracellular matrix which cover a central role in the angiogenesis and metastatic processes.[20,22,18]

Considering that heparanase activity releases heparan-sulfate fragments that stimulate the mitogenic activity of bFGF and other pro-angiogenic factors,[5] we were convinced of a possible antiheparanase activity of **FCE27266**.

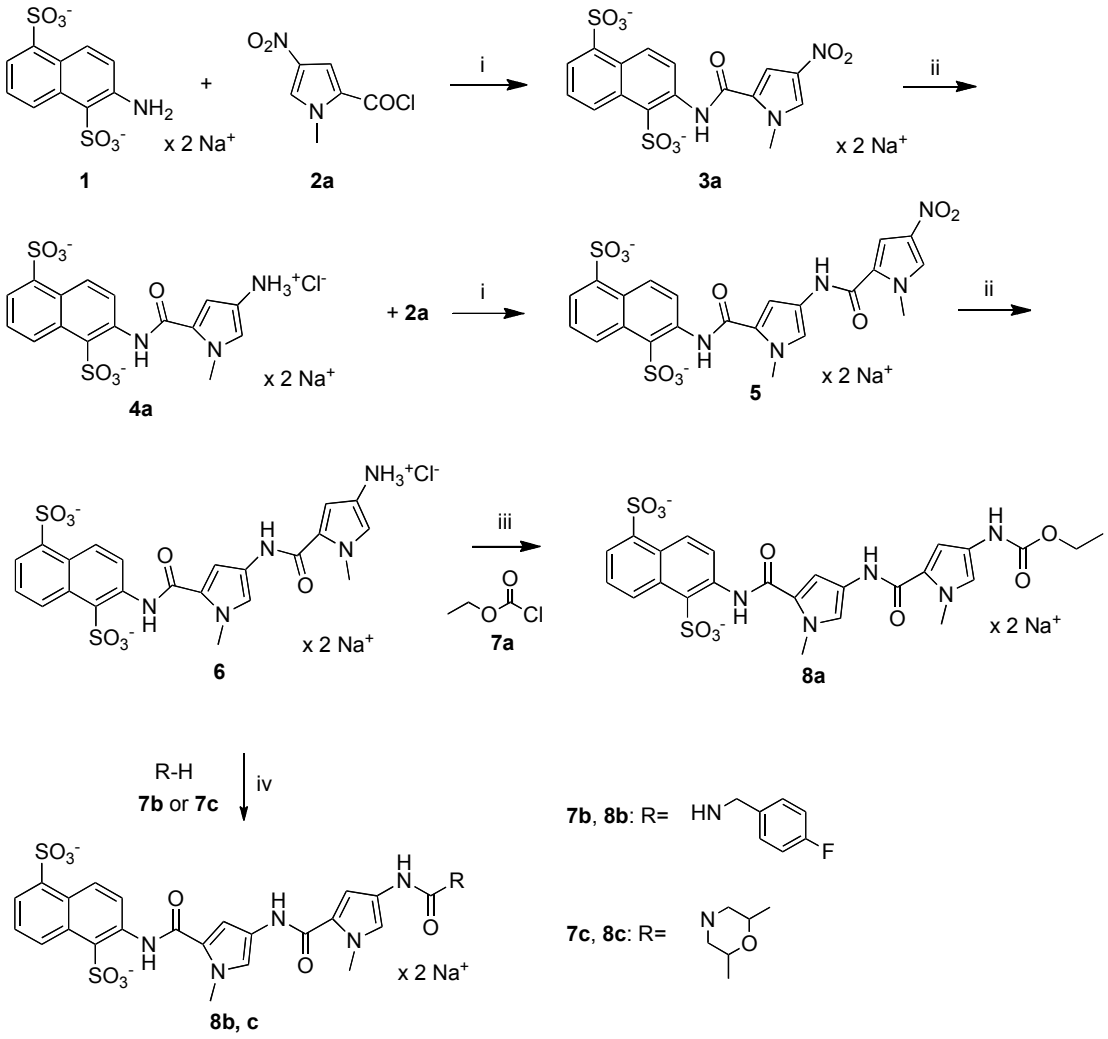
In this paper, we will describe our findings about the potent antiheparanase activity of **FCE27266**, as well as the development of a series of other structurally related compounds, which can be identified in the following chemical classes:

a) asymmetric ureas and carbamates. In the asymmetric ureas a portion of urea door the structure of **FCE27266** while the second portion carries a residue that can be

simply alkyl or aryl groups (compounds **8b,c**). In the carbamate **8a** the urea motif is replaced by a carbamate moiety.

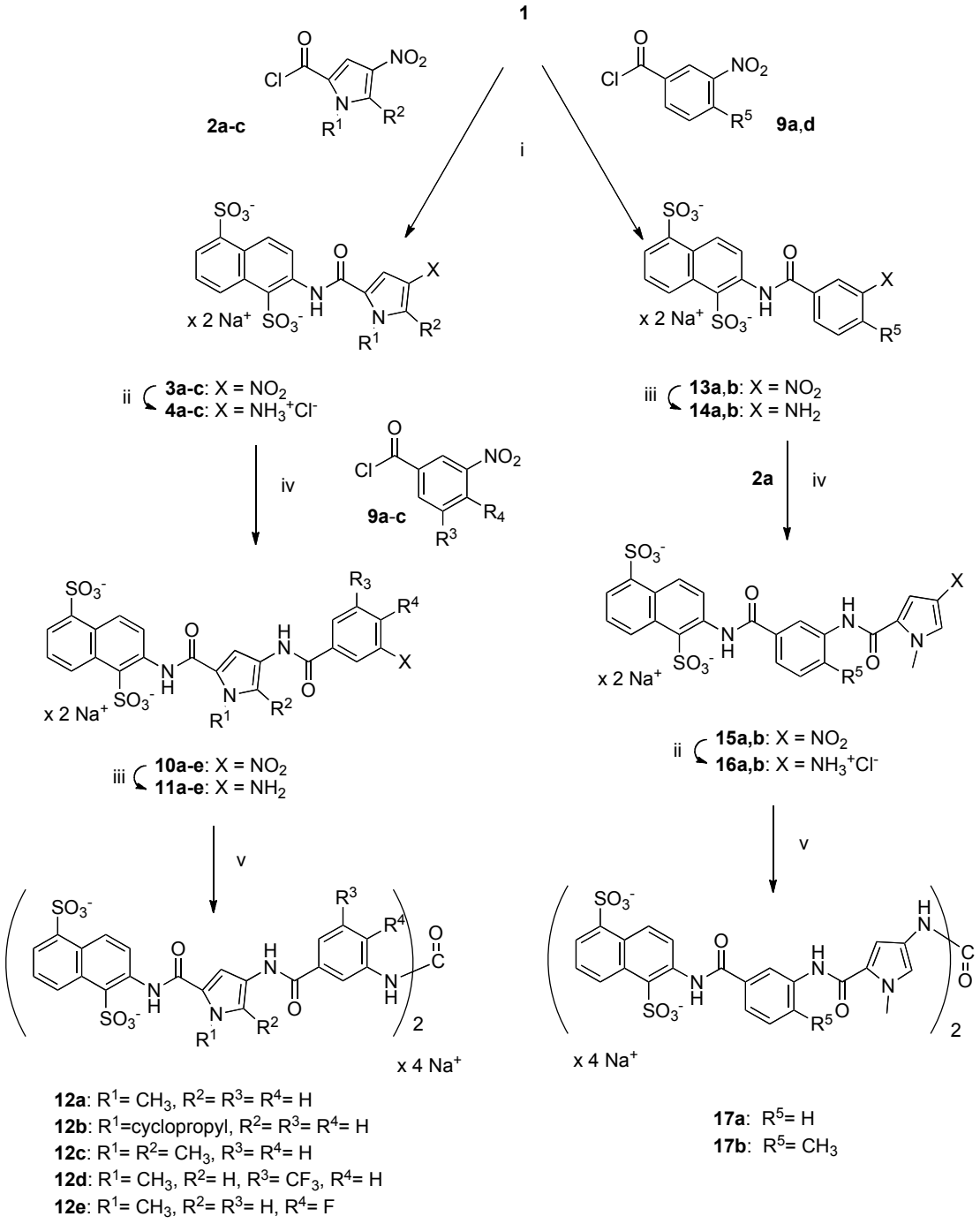
b) derivatives in which a pyrrole ring has been replaced with a benzene. The novel molecules resulted thus more similar to suramin while remaining a pyrrole residues that may be useful to maintain the certain degree of metabolic instability (compounds **12a, 17a,b**). At the same time, compounds bearing punctiform substituents such as a cyclopropane ring, methyl groups and fluorine atoms (**12b-e**) were also investigated. Both the methyl and cyclopropane substituents can modulate the lipophilicity as well as influence the free rotation of the amide linkages, and fluorine, as a bioisosteric replacement of hydrogen that, although induces only a slight steric perturbation, exhibits greater metabolic resistance and may have positive effects on interaction with target proteins.

The preparation of unsymmetrical compounds (Scheme 1) is straightforward and entails the acylation of the commercially available 2-amino naphthalene-1,5-disulfonic acid disodium salt **1** with the intermediate 1-methyl-4-nitro-pyrrole-2-carboxylic acid chloride **2a** affording in good yield the intermediate **3a**. The latter nitro derivative was easily reduced to the amine compound **4a** in quantitative yield by catalytic hydrogenation; from **4a**, repeating the sequence of coupling/reduction reactions with **2a**, similarly as described above, nitro derivative **5** and subsequently amine **6** were obtained. Carbamate **8a** was finally obtained by using ethyl chloroformate **7a**. Otherwise, unsymmetrical ureas **8b** and **8c** were easily obtained by condensation of **6** with 4-fluorobenzylamine **8b** or 2,6-dimethylmorpholine **8c** and 1,1′-carbonyldiimidazole (CDI) in 30 - 40% yield.



Scheme 1. Synthesis of asymmetric compounds **8a-c**. Reagents and conditions: (i) AcONa, H_2O/1,4-Dioxane, 5°C, 3h; (ii) H_2, 10% Pd/C, H_2O, HCl 1N, 4h; (iii) TEA, DMF dry, o.n; (iv) CDI, dry DMF, on, rt.

Modifications introducing 3-aminobenzoyl groups in place of the closest or farthest from urea couple of pyrroles are reported in Scheme 2 (compounds **12a-e** and **17a,b**). Among compounds **12a-e**, various substitutions were introduced at remaining pyrrole (compounds **12b,c**) or benzoyl rings (compounds **12a,d,e**). The amino intermediates **4a-c** were obtained using procedures as described in Scheme 1, with the appropriate pyrrole moieties **2a-c**, and the condensation with a nitrobenzoic acid chloride derivative (**9a-c**) gave the nitro compounds **10a-e** in 70-90% yield. Subsequent catalytic hydrogenation and final condensation (10 to 20% yield) by means of phosgene gave the urea derivatives **12a-e**.



Scheme 2. Synthesis of symmetric compounds **12a-e** and **17a,b**. Reagents and conditions: (i) AcONa, H_2O/1,4-Dioxane, 5°C, 3h; (ii) H_2, Pd/C 10%, H_2O, HCl 1N, 4h; (iii) H_2, Pd/C 10%, H_2O, 4h; (iv) AcONa, H_2O/Dioxane, 5°C, 3h or Na_2CO_3 5%, H_2O/toluene, 3h, rt (for **10a**); (v) $COCl_2$ 20% in toluene, AcONa, H_2O/1,4-dioxane, 0°C, overnight.

Similarly, compounds **17a,b** were synthesized by condensation of commercial **1** with 3-nitrobenzoyl chloride **9a** or 4-methyl-3-nitrobenzoyl chloride **9d** to yield the nitro derivatives **13a,b** which were easily reduced by catalytic hydrogenation to amines **14a,b** in quantitative yield. The latter amines reacted with 1-methyl-4-nitropyrrole-2-carboxylic acid chloride **2a** to give the nitro derivatives **15a,b** in 70-80% yield. Catalytic hydrogenation and final condensation with phosgene gave the urea derivatives **17a,b**.

All new synthesized compounds, including **FCE27266**, were investigated to assess their ability to inhibit the heparanase enzyme (Table 1). The assay is based on the cleavage of the synthetic pentasaccharide Fondaparinux (AGA*IA), in which the disaccharide product is determined colorimetrically.[23]

The investigation confirmed our hypothesis regarding the antiheparanase inhibitory activity of **FCE27266**, which showed values of inhibitory activity at low micromolar level in the AGA*IA test (IC_{50} 2.0 μM). Considering that the IC_{50} inhibitory activity of suramin is 26.6 μM (as reference compound evaluated in the same experimental set), while the most active analogue of suramin does not reach the value of 20 μM, the result appears quite surprising, far more important than our expectation, suggesting perhaps alternative or additional hypothesis of the mechanism of action of **FCE27266**.

Table 1. AGA*IA assay, results of compounds with IC_{50} < 10 μM.

Compd	AGAI*A (IC_{50}; μM)[a]
FCE27266	2.00
12a	0.86
12b	1.72
12c	3.40
12d	0.66
12e	8.53
17a	1.48
17b	5.64

[a] Values represent a mean of three determinations, with SD < 15%.

In contrast, the simple asymmetric ureas **8b,c** and carbamate **8a** proved completely devoid of activity.

An additional important result was obtained when a pyrrole ring of **FCE27266** was replaced by a benzene unit such as in compounds **12a** and **12c**. The novel structural change makes the compounds structurally more similar to suramin than distamycin and improving potently the activity as for compound **12a** which resulted three times more active than **FCE27266** in the AGA*IA assay. The dipyrroleurea **17a** was a bit less active than **FCE27266**, however proving more active than suramin.

Additionally, the introduction of methyl or trifluoromethyl groups as in compound **12c**, **17b** and **12d,e** significantly depresses the activity, while the cyclopropane ring as in compound **12b** decreases only slightly the activity.

Additional investigations were conducted only with molecules found active in AGA*IA assay (**12a-e**, **17a,b**).

A putative antiproliferative activity, against HT1080 (human fibrosarcoma), U87MG (human glioblastoma astrocytoma) and U2OS (human osteosarcoma) cell lines, was investigated by a SRB Cell Cytotoxicity Assay, based upon the quantitative staining of cellular proteins by sulforhodamine B (SRB).[24] For all compounds, on the three tumor cell lines, until a concentration of 2.5 μM, for 72h, the cell growth inhibition was less than 50%. The inhibition properties for invasion and cell adhesion, on HT1080 human cell lines, at the concentration of 2.5 μM, for a few of selected compounds, were also explored (data not shown). Compound **17a** resulted the most interesting compound with an inhibition of invasion more than 90% associated at a weak inhibition of adhesion, too.

To conclude, **17a** was also evaluated versus **FCE27266**, in a Real-Time Quantitative PCR (qPCR) on HT1080 (human fibrosarcoma) cells (Table 2), for the effect on the tumor transcription of genes encoding for some factors (FGF1/2, VEGF, MMP-9, HSPE-1). Against a weak effect on the proangiogenic factors (FGF and VEGF) and on Matrix metallopeptidase 9 (MMP9), both compounds showed a potent effect on the expression of heparanase (HPSE-1).

Table 2. Results with qPCR of **17a** vs **FCE27266**, tests at the concentration of 2.5 μM.

	Gene Expression (% mRNA vs. control)[a]				
	FGF-1	FGF-2	VEGF	MMP-9	HPSE-1
Control	~ 100	~ 100	~ 100	~ 100	~ 100
FCE27266	~ 44	~ 86	~ 100	~ 100	~ 1
17a	~ 87	~ 98	~ 85	~ 80	~ 4

[a] Values represent a mean of two determinations.

In summary **FCE27266** resulted a potent inhibitor of heparanase, about 10 times more active than suramin. The confirm of such hypothesis may give reason for its antimetastatic, even more than antiangiogenic activity, the latter being poor, as evidenced by the results of qPCR assay. Moreover, we found that some novel analogues of **FCE27266,** which are chemically similar to suramin**,** where a pyrrole ring is replaced with a benzene, **12a** (SST0548NA1) and **12d** (SST0613NA1), have higher activity in the heparanase inhibition assay. Finally, compound **17a** (SST0546NA1) showed the best overall profile, even superior than **FCE27266**, suggesting that this compound deserves to be further investigated for its potential antiangiogenic and antimetastatic properties as well as for any pharmacological application where a heparanase inhibitor may find a therapeutic indication.

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Supplementary data

Supplementary data (methods of biological assays and of chemical synthesis of most active tested product) associated with this article can be found, in the online version, at

References

1. Bernfield M, Gotte M, Park PW, Reizes O, Fitzgerald ML, Lincecum J, Zako M. *Annu Rev Biochem*. 1999; 68: 729-777.
2. Iozzo RV, San Antonio JD. *J Clin Invest.* 2001; 108: 349-355.
3. Kjellen L, Lindahl U. *Annu Rev Biochem*. 1991; 60: 443-475.
4. Pisano C, Vlodavsky I, Ilan N, Zunino C. *Biochem Pharmacol*. 2014; 89: 12-19.
5. Ilan N, Elkin N, Vlodavsky I. *Int J Biochem Cell Biol*. 2006; 38: 2018-2039.
6. Fux L, Ilan N, Sanderson RD, Vlodavsky I. *Trends Biochem Sci*. 2009; 34: 511-519.
7. Parish CR, Freeman C, Ziolkowski AF, He YQ, Sutcliffe EL, Zafar A, Rao S, Simeonovic CJ. *Matrix Biol.* 2013; 32: 228–233.
8. Ramani VC, Purushothaman A, Stewart MD, Thompson CA, Vlodavsky I, Au JL, Sanderson RD. *FEBS J*. 2013; 280: 2294–2306.
9. Vlodavsky I, Blich M, Li JP, Sanderson RD, Ilan N. *Matrix Biol*. 2013; 32: 241– 251.
10. Yang Y, Ren Y, Ramani VC, Nan L, Suva LJ, Sanderson RD. *Cancer Res*. 2010; 70: 8329–8338.
11. Ferro V, Hammond E, Fairweather JK. *Mini Rev Med Chem*. 2004; 4: 693-702.
12. Jia L, Ma S. *Eur J Med Chem*. 2016; 121: 209-220.
13. Rivara S, Milazzo FM, Giannini G. *Future Med Chem*. 2016; 8: 647-680.
14. Casu B, Vlodavsky I, Sanderson RD. *Pathophysiol Haemost Thromb*. 2008; 36: 195-203.
15. Nakajima M, DeChavigny A, Johnson CE, Hamada J, Stein CA, Nicolson GL. *J Biol Chem.* 1991; 266: 9661-9666.
16. Marchetti D, Reiland J, Erwin B, Madhuchhanda R. *Int J Cancer.* 2003; 104: 167-174.
17. Ciomei, M.; Pastori, W.; Mariani, M.; Sola, F.; Grandi, M.; Mongelli, N. New sulfonated distamycin a derivatives with bFGF complexing activity. *Biochem. Pharmacol*. **1994**, *47*, 295-302.
18. Sola F, Farao M, Ciomei M, Pastori A, Mongelli N, Grandi M. *Invasion Metastasis.* 1995; 15: 222-231.

19. Finch PW, Yee LK, Chu MYW, Chen TM, Lipsky MH, Maciag T, Friedman S, Epstein MH, Calabresi P. *Pharmacology*. 1997; 55: 269-278.
20. Manetti F, Cappello V, Botta M, Corelli F, Mongelli N, Biasoli G, Lombardi Borgia A, Ciomei M. *Bioorg Med Chem*. 1998; 6: 947-958.
21. Schneider GP, Salcedo R, Dong HF, Kleinman HK, Oppenheim JJ, Howard OMZ. *Clin Cancer Res*. 2002; 8: 3955-3960.
22. Lozano RM, Jimenez MA, Santoro J, Rico M, Gimenez-Gallego G. *J Mol Biol.* 1998; 281: 899-915.
23. Hammond E, Li CP, Ferro V. *Anal Biochem.* 2010; 396: 112-116.
24. Vichai V, Kirkitara K. *Nat Protoc*. 2006; 1: 1112-1116.