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2,3-Dihydro-1,3-dioxo-1*H*-isoindole-5-carboxylic acid derivatives: a novel class of small molecule heparanase inhibitors

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Abstract—A novel class of 2,3-dihydro-1,3-dioxo-1*H*-isoindole-5-carboxylic acids are described as inhibitors of the *endo*-β-glucuronidase heparanase. Several of the compounds, for example, 2-[4-propylamino-5-[5-(4-chloro)phenyl-benzoxazol-2-yl]phenyl]-2,3-dihydro-1,3-dioxo-1*H*-isoindole-5-carboxylic acid (**9c**), display potent heparanase inhibitory activity (IC_{50} 200–500 nM) and have high selectivity (>100-fold) over human β-glucuronidase. They also show anti-angiogenic effects. Such compounds should serve as useful biological tools and may provide a basis for the design of novel therapeutic agents.

Heparanase is an *endo*-β-glucuronidase that cleaves heparan sulfate and is implicated in diverse physiological and pathological processes.[1,2] Expression of heparanase has long been correlated with the metastatic potential of tumour cells and with angiogenesis.[3–7] In animal models treatment with heparanase inhibitors has been shown to markedly reduce the incidence of metastasis.[8] Heparanase thus offers an attractive drug target but progress in this area has been limited by the currently available repertoire of inhibitors. The most advanced inhibitor is PI-88,[8] which is currently in Phase II clinical trials, has as its principal component the highly sulfated pentasaccharide derivative (**1**). However, heparanase inhibition is only one component of the multifunctional profile of this compound, potentially complicating an understanding of its mode of action. Iminosugar related heparanase inhibitors of moderate potency have also been described but showed greater potency towards mammalian β-glucuronidase and also contain potentially labile functionality.[9,10] Weak heparanase inhibitory activity has been reported for an aza-pseudodisaccharide[11] and for the natural product trachyspic acid.[12] We now report on a series of potent and selective small molecule inhibitors, which may provide useful biological tools for unravelling the complex biology of heparanase and which may also serve as basis for the design of novel therapeutic agents.

High throughput screening identified the 2,3-dihydro-1,3-dioxo-1*H*-isoindole-5-carboxylic acid (**2a**) as a small molecule inhibitor of heparanase (IC_{50} 8 μM) with modest anti-angiogenic activity (IC_{50} 40 μM). The molecular weight, lipophilicity and polar surface area of this compound together with synthetic accessibility of related structures were deemed acceptable as the basis

Keywords: Heparanase; β-Glucuronidase; Angiogenesis; Metastasis; Isoindole-5-carboxylic acid.
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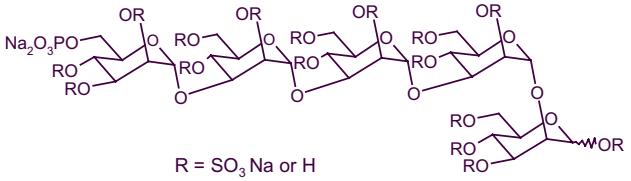

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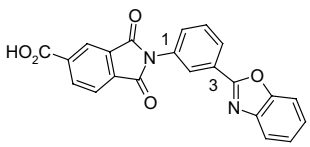


(2a)

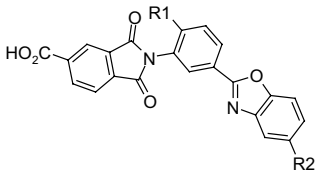
for a programme directed to identifying more potent and selective heparanase inhibitors. Compounds were evaluated for their heparanase inhibitory activity[13] and their ability to inhibit angiogenesis.[14]

Early investigations demonstrated that the carboxylic acid group was crucial for the inhibitory activity; its removal or esterification abolished heparanase inhibitory activity. Furthermore, a 1,4-disposition of the isoindole-5-carboxylic acid moiety and the pendant benzoxazole ring system also resulted in a fall in inhibitory activity.

Efforts were therefore focussed on substitution of both the central aromatic core and the benzoxazole moiety of (**2a**) while retaining the other structural features. Access to such compounds was via the general route summarised in Scheme 1. The *m*-nitrobenzoic acids (**3**) were first converted to the *o*-hydroxybenzamides, cyclisation of which provided the intermediate benzoxazoles (**4**). Reduction of the nitro group followed by treatment with 1,3,5-benzenetricarboxylic anhydride gave the target compounds (**2a**–**h**). This short series of compounds, which included 2-methoxy substitution in the central phenyl ring led to the selection of the 5-phenyl derivative (**2h**) (OGT2492) for further optimisation (Table 1). Although this compound represented only a slight improvement in heparanase inhibition over (**2a**) it did display much improved activity in the cell-based anti-angiogenic assay. It also showed no inhibitory activity against human β-glucuronidase at 100 μM. In a corroborative assay, OGT2492 inhibited the cleavage of heterologous FITC-labelled heparan sulfate (as assessed by size exclusion HPLC) with an IC_{50} similar to that obtained via the bFGF protocol.[13]

In order to explore the influence of substitution on the pendant phenyl ring the synthetic route was slightly modified (Scheme 2) to include a boronic acid coupling step on the central 5-bromobenzoxazole intermediate (**5**). Introduction of fluoro or CF_3O substitution, for example, (**6b**), (**6h**) and (**6q**), led to a significant increase

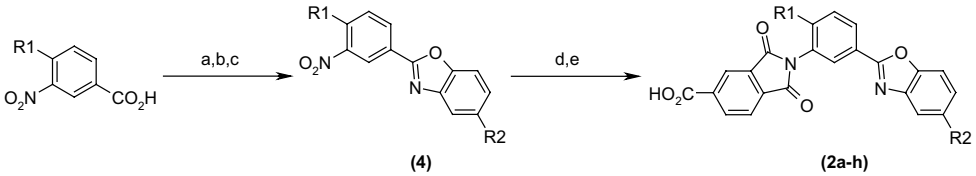
Table 1. In vitro inhibition of heparanase and anti-angiogenic activity of (**2a**–**h**)



Compound	R1	R2	Heparanase inhibition[a] IC_{50} (μM)	Angiogenesis inhibition[a] IC_{50} (μM)
2a	H	H	8.0	40
2b	H	Cl	1.5	*
2c	H	Me	2.5	>50
2d	H	Ph	3.0	5
2e	MeO	H	>10	NT
2f	MeO	Cl	3.0	*
2g	MeO	Me	>10	NT
2h	MeO	Ph	3.0	2

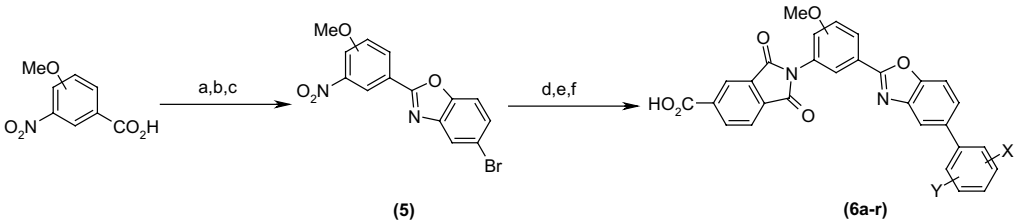
*Poor solubility precluded testing; NT: not tested.
[a] Mean of at least two determinations.

(IC_{50} 0.4 μM) in heparanase inhibitory activity (Table 2). A loss in activity was observed with the introduction of an *ortho*-methoxy group (**6l**) and with a more polar (e.g., acetamide **6k**) group. Translocating the methoxy substituent on the central phenyl ring to the 4-position had mixed effects on heparanase inhibitory activity depending upon the substitution pattern of the pendant phenyl ring. Noteworthy was the methylenedioxy derivative (**6p**) (IC_{50} 0.2 μM), one of the most potent inhibitors in this series that was also active in the angiogenesis assay (IC_{50} 3 μM). Although there was a poor correlation between anti-angiogenic activity and heparanase inhibitory activity, particularly striking was the potent anti-angiogenic activity (IC_{50} 0.75 μM) of the 3′,5′-difluoro derivative (**6i**). Monitoring activity against human β-glucuronidase showed, inter alia, that (**6p**) and (**6q**) showed no inhibition at 100 μM.



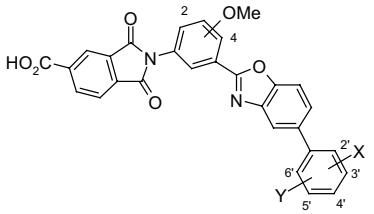
(4) (2a-h)

Scheme 1. Reagents and conditions: (a) $(COCl)_2$, DMF, THF; (b) *o*-aminophenol, THF; (c) TsOH, toluene, reflux; (d) H_2, Pd/C, EtOAc; (e) 1,3,5-benzenetricarboxylic anhydride, AcOH, reflux.



Scheme 2. Reagents and conditions: (a) $(COCl)_2$, DMF, THF; (b) *o*-aminophenol, THF; (c) TsOH, toluene, reflux; (d) $ArB(OH)_2$, $Pd(PPh_3)_4$, Na_2CO_3, dioxane, 100 °C; (e) H_2, Pd/C, EtOAc; (f) 1,3,5-benzenetricarboxylic anhydride, AcOH, reflux.

Table 2. In vitro inhibition of heparanase and anti-angiogenic activity of (**6a–r**)



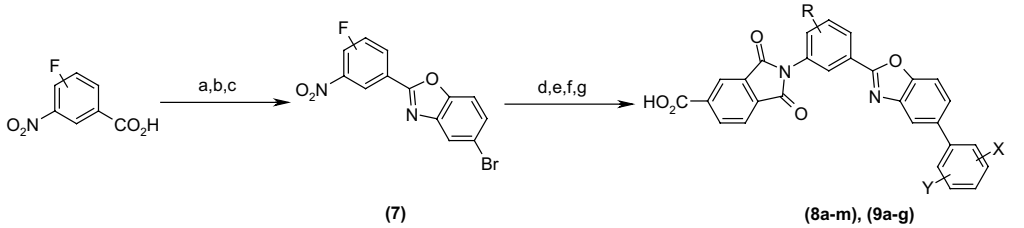
Compound	R	Phenyl substitution: X, Y	Heparanase inhibition[a] IC_{50} (μM)	Angiogenesis inhibition[a] IC_{50} (μM)
6a	2-MeO	3′-F	1.0	>20
6b	2-MeO	4′-F	0.4	15
6c	2-MeO	3′-Cl	0.6	7
6d	2-MeO	4′-Cl	4.5	20
6e	4-MeO	4′-Cl	2.5	NT
6f	2-MeO	2′,4′-di-F	0.8	12
6g	2-MeO	3′,4′-di-F	0.6	20
6h	2-MeO	3′,5′-di-F	0.4	>20
6i	4-MeO	3′,5′-di-F	0.9	0.75
6j	2-MeO	3′-CN	3.0	20
6k	2-MeO	3′-NHCOMe	9.0	NT
6l	2-MeO	2′-MeO	9.0	NT
6m	2-MeO	3′-MeO	3.0	15
6n	2-MeO	4′-MeO	0.45	15
6o	2-MeO	3′,4′-OCH_2O	0.75	>20
6p	4-MeO	3′,4′-OCH_2O	0.2	3
6q	2-MeO	4′-OCF_3	0.4	20
6r	4-MeO	4′-OCF_3	>10	NT

NT: not tested.
[a] Mean of at least two determinations.

The limited solubility of several members of the compounds listed in Table 2 led us to introduce potentially solubilising substitutions, for example, $O(CH_2)_2OMe$, $O(CH_2)_2NMe_2$, $NH(CH_2)_2OMe$. Access to these systems was straightforward and based upon a range of nucleophilic displacements on the core intermediate (**7**) (Scheme 3), followed by arylboronic acid coupling. From the derivatives evaluated (Table 3) certain SAR becomes apparent: within a short series of amino substitutions $NHPr^n$ (**8j**) was optimal and notably the NHMe (**8h**) was poorly active. Furthermore, although (**8j**) and (**8k**) demonstrated good heparanase inhibitory activity, their anti-angiogenic activity fell away markedly. N-Methylation of (**8j**) to give the corresponding tertiary amine (**8l**) resulted in a >20-fold drop in activity. Heparanase inhibitory activity for the short series of ethers at the 4-position (**8c–g**), including those incorporating an additional heteroatom, was reduced with increasing bulk of the substituent.

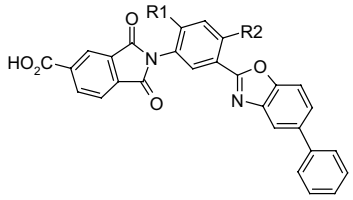
With the 4-$NHPr^n$ fixed, further optimisation was explored via introduction of substitutions in the pendant phenyl ring (Table 4). The 4′-F and 4′-Cl substituents gave potent heparanase inhibitors and, significantly, most of these compounds showed appreciable activity in the angiogenesis assay. Several of the 4-$NHPr^n$ derivatives were tested for inhibition of human β -glucuronidase with similar results to earlier analogues, that is (**8j**), (**9a**) and (**9c**) showed no inhibition at 100 μM. Overall, compounds such as (**9a**) and (**9c**) displayed the best combination of in vitro activity and solubility.

Some compounds reported in this study display a poor correlation between heparanase activity and anti-angiogenic activity. There are several possible reasons for this: poor solubility of test compounds, high protein binding, poor cellular uptake or an off-target component to this activity. Certainly, the analogues reported in Table 4 showed improved solubility and this might explain their efficacy in the angiogenesis assay. In summary, the data presented here describes the first series of potent, small molecule heparanase inhibitors that show excellent selectivity over human β-glucuronidase. As



Scheme 3. Reagents and conditions: (a) $(COCl)_2$, DMF, THF; (b) *o*-aminophenol, THF; (c) TsOH, toluene, reflux; (d) NHRR′, NaH, THF; (e) $ArB(OH)_2$, $Pd(PPh_3)_4$, Na_2CO_3, dioxane, 100 °C; (f) H_2, Pd/C, EtOAc; (g) 1,3,5-benzenetricarboxylic anhydride, AcOH, reflux.

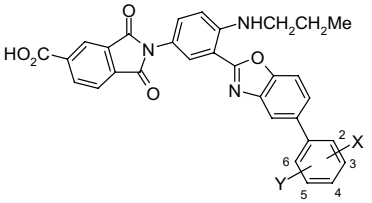
Table 3. In vitro inhibition of heparanase and anti-angiogenic activity of (**8a**–**m**)



Compound	R1	R2	Heparanase inhibition[a] IC_{50} (μM)	Angiogenesis inhibition[a] IC_{50} (μM)
8a	OEt	H	3.0	>20
8b	$O(CH_2)_2OMe$	H	4.0	5
8c	H	OEt	1.3	>20
8d	H	OPr^i	3.0	10
8e	H	OBu^t	>10	NT
8f	H	$O(CH_2)_2OMe$	5.0	10
8g	H	$O(CH_2)_2NMe_2$	>10	NT
8h	H	NHMe	>10	NT
8i	H	NHEt	1.0	>20
8j	H	$NHPr^n$	0.25	15
8k	H	$NHBu^n$	0.4	10
8l	H	$NMePr^n$	6.0	NT
8m	H	$NH(CH_2)_2OMe$	2.0	20

NT: not tested.
[a] Mean of at least two determinations.

Table 4. In vitro inhibition of heparanase and anti-angiogenic activity of (**9a**–**g**)



Compound	Phenyl substitution: X, Y	Heparanase inhibition[a] IC_{50} (μM)	Angiogenesis inhibition[a] IC_{50} (μM)
9a	4′-F	0.5	0.25
9b	3′-Cl	2.5	0.75
9c	4′-Cl	0.2	1
9d	3′,5′-di-F	3.0	NT
9e	3′-Cl, 4′-F	2.0	1
9f	4′-CF_3	2.0	0.2
9g	4′-OCF_3	0.8	0.2

NT: not tested.
[a] Mean of at least two determinations.

such they should serve as useful biological tools and may provide a basis for the design of novel therapeutic agents.[15]

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13. Heparanase assay: The assay is based upon the use of the specific binding of basic fibroblast growth factor (bFGF) to heparan sulfate. Heparan sulfate can be detected via binding of bFGF using a horse radish peroxidase-conjugated bFGF antibody. Following cleavage of high molecular weight heparan sulfate by heparanase, the smaller material generated will no longer adhere to the surface of a 96 well plate and hence heparanase activity can be followed as a reduction in bFGF binding. Nunc Maxisorp 96 well plates are coated for 16 h at rt with 100 μL/well

0.04 mg/mL heparan sulfate in PBS. The wells are then aspirated and blocked for 1 h with 200 μL/well 1% BSA–PBS. Following five washes with 0.01% BSA, 0.05% Tween20 PBS (wash buffer), 100 μL of recombinant human basic FGF (90 ng/mL in 0.1% BSA/PBS) is added per well and the plate is incubated at room temperature for 1 h. After a further five washes with the wash buffer, 10 μL of test compound (in 10% DMSO) and 90 μL of human heparanase in 100 mM sodium acetate, 5 mM $CaCl_2$, pH 5.5 are added to each well and the plate incubated for 2 h at 37 °C. The wells are washed again with wash buffer and 100 μL of bFGF antibody-horse radish peroxidase conjugate added. The plate is incubated at room temperature for 1 h and washed again five times with wash buffer. TMB peroxidase substrate (100 μL) is added and the colour allowed to develop for 10 min. The reaction is stopped with 50 μL 1 M H_2SO_4 and the colour read at 450 nm on a plate reader.

14. Angiogenesis assay: A commercial angiogenesis assay for analysing the angiogenic or anti-angiogenic properties of test compounds (AngioKit catalogue no ZHA-1000, TCS CellWorks Ltd, Buckingham, UK) was used. In this assay, human endothelial cells were co-cultured with other human cells in a specifically designed medium. The endothelial cells initially form small islands within the culture matrix. They subsequently proliferate and then enter a migratory phase during, which they move through the matrix to form threadlike tubule structures. These gradually join up (by 12–14 days) to form networks of anatomising tubules, which closely resemble a capillary bed structure. These tubules stain positive for von Willebrand's Factor, Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1 or CD31) and Intercellular Adhesion Molecule-2 (ICAM-2). The assay was supplied as growing cultures at the earliest stage of tubule formation in a 24 well plate format. It is designed so that test compounds and conditioned media can be added to the cultures within individual wells and the resulting effect on tubule formation can then be monitored. Positive and negative test agents, for example, vascular endothelial growth factor (VEGF) and suramin, are included. On day 1, fresh growth medium, medium plus control agent or medium plus test compound was added to the cells and the cultures were incubated at 37 °C, 5% CO_2. Test compounds were dissolved in DMSO and the final concentration of DMSO in the medium did not exceed 0.1% (v/v). The specified medium was changed at days 4, 7 and 9 and the cells were monitored for growth. On day 11, the cells were washed with Dulbecco's phosphate-buffered saline (PBS) and fixed using 70% ethanol (−20 °C) for 30 min at room temperature. After fixing, the cells were washed and treated with blocking buffer, 1% BSA in PBS. The cells were stained for PECAM-1 on the same day, following standard immunohistochemistry procedures using mouse anti-human CD31 as the primary antibody and a goat anti-mouse IgG alkaline phosphate conjugate. Tubule formation was quantitatively assessed by measuring PECAM-1 positive staining using the image analysis program 'Matrox inspector' to evaluate the percentage tubule staining relative to an untreated control.
15. All final products were characterised by ^1H NMR and MS.