Phenotypic Screening To Discover Novel Chemical Series as Efficient Antihemorrhagic Agents

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Supporting Information

ABSTRACT: In an effort to find novel chemical series as antifibrinolytic agents, we explore α -phenylsulfonyl- α -spiropiperidines bearing different zinc-binding groups (ZBGs) to target those metalloproteinases involved in the fibrinolytic process: MMP3 and MMP10. Surprisingly, all these new chemical series were inactive against these metalloproteinases; however, several new molecules retained the antifibrinolytic activity in a phenotypic functional assay using thromboelastometry and human whole blood. Further optimization led to compound **38** as a potent antifibrinolytic agent in vivo, three times more efficacious than the current standard-of-care (tranexamic acid, TXA) at 300 times lower dose. Finally, in order to decipher the underlying mode-of-action leading to this phenotypic response, an affinity-based probe **39** was successfully designed to identify the target involved in this response: a potentially unknown mechanism-of-action in the fibrinolytic process.

KEYWORDS: Phenotypic screening, novel antifibrinolytic agents, affinity-based probe

Fibrinolysis is necessary to prevent blood clots from growing when coagulation is activated to block a hemorrhage.¹ Nevertheless, hyperfibrinolysis can result in excessive bleeding, and antifibrinolytic agents are indispensable to control blood loss after trauma episodes or in major surgeries, for example.² To date, two synthetic antifibrinolytics are commercially available for clinical use, tranexamic acid (**1**, Figure 1A) and ϵ -aminocaproic acid. Additionally, aprotinin (a protease inhibitor derived from bovine lung) was effectively used to reduce bleeding during complex surgery. However, this drug was associated with adverse side effects,³ and it was temporarily abandoned in 2008 except for a limited number of indications in Europe and Canada.⁴ Important side effects have also been associated with tranexamic acid,⁵ and ϵ -aminocaproic acid is not as effective.⁶ Thus, discovery of more potent and safer antifibrinolytics represents a real medical challenge.

Figure 1. (A) Chemical structure of known antifibrinolytic tranexamic acid (**1**, TXA). (B) Chemical structure of our preclinical candidate CM-352 (**2**). (C) Exploration around chemical structure **3** for new antihemorrhagic agents.

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Recent studies have evidenced that matrix metalloproteinases (MMPs) are associated with the fibrinolytic system regulating thrombus dissolution. MMPs can proteolyze fibrin and other components of the fibrinolytic system^{7,8} and have been proposed to play a role in hemorrhage, degrading basement membrane and disregulating blood–brain barrier (BBB) after stroke^{9,10} and other cerebral pathologies such as traumatic brain injury.¹¹ All together, these data indicate that MMPs are potential targets for pharmacological modulation in these pathologies, and inhibition of MMPs has emerged as a new pharmacological strategy for bleeding control. In this regard, we have recently developed the preclinical candidate CM-352 (**2**) for the acute treatment of bleeding (Figure 1B), a new antifibrinolytic agent capable of inhibiting MMP10 and MMP3 with an optimal safety and pharmacokinetic profile as well as with an outstanding *in vivo* efficacy.^{12–14} This molecule **2** contains a hydroxamic acid moiety as a zinc chelator. This zinc-binding group (ZBG), especially represented by MMP inhibitors containing it, has been associated with potential toxicity and reversible adverse side effects, principally in the musculoskeletal system, after long-term administration.^{15,16} In recent years, this fact has encouraged many researchers to study different alternatives to this ZBG and several options have been described.^{17–25}

In our case, this toxicity is not a problem because our MMP inhibitors have been designed for an acute treatment of hyperfibrinolysis (not chronic), and our preclinical candidate **2** has been demonstrated to be safe in animal models.^{12,14} Nevertheless, as a back-up strategy, we decided to explore different ZBGs to hydroxamic acid moiety. We designed alternative chemical series, and herein, we present our new antihemorrhagic agents that surprisingly were inactive against matrix metalloproteinases but showed antifibrinolytic activity. In addition, to decipher the underlying mode-of-action (MoA) leading to this phenotypic response, we designed and synthesized an affinity-based probe to identify the potentially unknown targets involved in this fibrinolytic process.

Our previously described studies regarding MMP inhibition exhibited α -phenylsulfonyl- α -spiropiperidine as a good scaffold to obtain potent pan-MMP inhibitors.¹³ Hence, we decided to design a new series of inhibitors based on structure **3** (Figure 1C), replacing the hydroxamic acid of our preclinical candidate **2** by different ZBG and modulating the activity exploring substitution at the phenyl ring and at the nitrogen atom of the piperidine. This exploration also covered a broad range of values across key physicochemical properties, *in silico* predicted (Table S5, Supporting Information).

As a first exploration, we decided to prepare compounds with a 4-trifluoromethoxyphenyl substituent at the 4-position of the phenyl ring as we had previously observed that this was an optimal group for yielding potent MMP3 and MMP10 inhibitors with optimal antifibrinolytic activity (e.g., previously described compound **4** with a hydroxamic acid as ZBG, Table 1).¹³ Thus, compounds with different ZBGs (**7–20**) were synthesized as well as two compounds with a *N,O*-dimethylhydroxamate group (**5**) and a hydrogen atom (**6**) as negative controls, expected to be inactive versus MMPs and with no antifibrinolytic effect (details for synthetic routes described in Supporting Information). Inhibitory activities of new compounds **5–20** and previously described hydroxamate **4** against MMP3 and MMP10²⁶ are shown in Table 1. As expected, the replacement of hydroxamic acid by an *N,O*-dimethylhydroxamate group (compound **5**) or its elimination

Table 1. Exploration of Different ZBGs

Cmpd	R	MMP3 IC ₅₀ (nM) ^b	MMP10 IC ₅₀ (nM) ^b	TE EC ₅₀ (nM)
4^a		4 ^a	4 ^a	≤ 400 ^a
5		>10000	>10000	3700 ^c
6	H	>10000	>10000	>15000 ^b
7		>10000	>10000	1800 ^c
8		>10000	>10000	>15000 ^b
9		>10000	>10000	>15000 ^b
10		>10000	>10000	900 ^c
11		>10000	>10000	>15000 ^b
12		>10000	>10000	3700 ^c
13		>10000	>10000	>15000 ^c
14		>10000	>10000	>15000 ^b
15		>10000	>10000	7500 ^c
16		>10000	>10000	900 ^c
17		>10000	>10000	>15000 ^b
18		>10000	>10000	≤ 400 ^c
19		>10000	>10000	>15000 ^b
20		>10000	>10000	>15000 ^b

^aCompound and data previously reported in ref 13. ^bAssay was performed in duplicate. ^cAssay was performed in triplicate. Cmpd, compound; TE, thromboelastometry.

(compound **6**) resulted in inactive derivatives against these two matrix metalloproteinases, but all the other compounds with

Table 2. Exploration of Different Substituents at the Phenyl Ring

Cmpd	structure	R ₁	R ₂	R ₃	R ₄	MMP3 IC ₅₀ (nM) ^a	MMP10 IC ₅₀ (nM) ^a	TE EC ₅₀ (nM) ^b
21	1	H	H	O-4-OCF ₃ -Ph	Ph	>10000	>10000	≤400 ^b
22	1	H	H	O-4-OCF ₃ -Ph	F	>10000	>10000	>15000
23	1	H	H	O-4-OCF ₃ -Ph	OCH ₃	>10000	>10000	3700 ^b
24	1	H	H	O-4-OCF ₃ -Ph	2-thiophene	>10000	>10000	900 ^b
25	1	H	H	O-4-OCF ₃ -Ph	5-methyl-2-furyl	>10000	>10000	>15000 ^b
26	1	H	H	O-4-CONHCH ₃ -Ph	H	>10000	>10000	3700 ^b
27	1	H	H	NH-cyclohexyl	H	>10000	>10000	≤400 ^b
28	1	NH-cyclohexyl	H	H	H	>10000	>10000	3700 ^b
29	1	H	Cl	O-4-OCF ₃ -Ph	H	>10000	>10000	3700 ^b
30	1	H	Cl	OCH ₃	H	>10000	>10000	7500 ^b
31	1	H	Cl	O-4-OCH ₃ -Ph	H	>10000	>10000	>15000 ^a
32	1	H	Cl	OCH ₃	Ph	>10000	>10000	15000 ^b
33	2	H	H	NH-cyclohexyl		>10000	>10000	>15000 ^a
34	2	H	Cl	OCH ₃		>10000	>10000	3700 ^b
35	3	H	Cl	OCH ₃		>10000	>10000	>15000 ^b
36	3	H	Cl	O-4-OCH ₃ -Ph		>10000	>10000	3700 ^b

^a Assay was performed in duplicate. ^b Assay was performed in triplicate. Cmpd, compound; TE, thromboelastometry.

different ZBGs (carboxylic acid, esters, amides, ureas, etc.) did not show any activity either.

At this point and despite these negative results, we decided to test the compounds as antifibrinolytic agents in a phenotypic functional assay using human whole blood and thromboelastometry (TE) in the presence of tissue plasminogen activator (tPA).²⁶ Results, as the effective concentration of compound needed to delay clot lysis time by 50% (EC₅₀), are shown in Table 1. In this case, surprisingly, compound 5 with a *N,O*-dimethylhydroxamate resulted active in the low micromolar range, suggesting a potentially new MoA for the antifibrinolytic activity. Similar results were obtained for carboxylic acid 7. Nevertheless, 6 with a hydrogen atom and derivatives 8 and 9 with a methyl or benzyl ester attached to the α -spiropiperidine core resulted again in the abolishment of the antifibrinolytic activity. Better results were obtained with phenyl ester 10 with an IC₅₀ of 900 nM. Nevertheless, hydroxyl derivative 11 was inactive in the thromboelastometry assay. On the other hand, antifibrinolytic activity was conserved for primary amide (12), which exhibited similar potency compared to 5 and 7. A variety of *N*-substituted amides were also assayed, and we concluded that a piperazin-2-one group (15) and specially a thiazole (13) or a pyridyl group (14) as *N*-substituents are detrimental for the phenotypic activity.

On the other hand, compound 16 with a 2-aminophenyl amide exhibited an EC₅₀ in the high nanomolar range, but the antifibrinolytic activity disappeared when the amino group was replaced by a hydroxyl group as shown for 2-hydroxy-phenyl amide (17). Oppositely, a very potent antifibrinolytic agent was obtained with a *N*-methyl acylurea group as ZBG (18) with an EC₅₀ ≤ 400 nM. Finally, acylsulfonamides 19 and 20 were assayed, but these two compounds did not show any activity as antifibrinolytics.

Considering results from this phenotypic assay, a clear SAR was not observed, and we decided to continue further exploration around three of the most active ZBGs. Thus, we

selected benzyl ester (10), 2-aminophenyl amide (16), and *N*-methyl acylurea (18) as the most promising ZBGs and prepared derivatives with different substituents at the phenyl ring of the 2-aminophenyl amide moiety and at the phenyl ring attached to the α -spiropiperidine (see Supporting Information for synthetic details). These synthesized derivatives bearing different substituents at both phenyl rings were evaluated for their inhibitory activity against MMP3 and MMP10. Their EC₅₀s, in the phenotypic assay, were also determined (Table 2).

As happened with compounds described above, all these new molecules were inactive against these two matrix metalloproteinases but most of them retained their activity as antifibrinolytic agents (except derivatives 22, 25, 31, 33, and 35). These results confirmed that the antifibrinolytic activity was achieved through an unknown mechanism, different from the expected MMP inhibition pathway.

Regarding compounds with a 2-aminophenyl amide moiety as ZBG, we first assayed compounds with different substituents at the *para*-position of amino group. From them, compound 21 bearing a phenyl group appeared to be the most potent. Molecules with a thiophene ring (24) or a methoxy group (23) showed a similar or slightly decreased antifibrinolytic activity compared to 16 (unsubstituted 2-aminophenyl amide). However, compounds bearing a fluorine atom (22) or a 5-methylfuryl group (25) as a substituent resulted in inactive compounds.

Then, we focus our attention on the substitution at the phenyl ring attached to the α -spiropiperidine core, and we first evaluated compounds 26–31 with an unsubstituted 2-aminophenyl amide as ZBG. In this context, compound 26 with the same substitution pattern as our preclinical candidate 2 was assayed, but its potency was not as high as 16 with a 4-trifluoromethoxyphenyl substituent at the 4-position of the phenyl ring. Better results were obtained with compound 27 containing a cyclohexylamine group at 4-position of the phenyl

group. Nevertheless, the antifibrinolytic activity was reduced when this substituent was located at 2-position (**28**).

A decrease in antifibrinolytic activity was also observed for compounds with a chlorine atom at the 3-position of the phenyl ring (compounds **29–31**). Finally, we assayed compound **32** with a 2-aminophenyl amide substituted with a phenyl group at the *para*-position, but the same detrimental influence of chlorine atom was detected.

We next evaluated compounds **33** and **34** with a benzyl ester as ZBG. This time, a cyclohexylamine group at the 4-position of the phenyl group (compound **33**) did not improve the potency as occurred with 2-aminophenyl amide. Either compound **34** with a chlorine at the 3-position and a methoxy group at the 4-position resulted in an improved antifibrinolytic agent. Finally, derivatives **35** and **36** bearing an *N*-methyl acylurea as ZBG confirmed the negative influence of a chlorine atom at the 3-position of the phenyl ring; neither compound **35** (with a 4-methoxy at 4-position) nor compound **36** (with a 4-methoxyphenyl substituent) exhibited higher potency in the thromboelastometry assay than compound **18**.

We finally turned our attention to the basic nitrogen of the spiropiperidine and tertiary amines **37** and **38** with a propyl group was prepared (synthesis described in [Supporting Information](#)). As expected, these two compounds were inactive against MMP3 and MMP10; and **37**, bearing an *N*-methyl acylurea, was also inactive in the thromboelastometry assay ([Table 3](#)). Nevertheless, the antifibrinolytic activity was

Table 3. Exploration of *N*-Substituted Spiropiperidines

Cmpd	R	MMP3 IC ₅₀ (μM) ^a	MMP10 IC ₅₀ (μM) ^a	TE (nM)	EC ₅₀
37		>10000	>10000	>15000 ^a	
38		>10000	>10000	≤400 ^b	

^a Assay was performed in duplicate. ^b Assay was performed in triplicate. Cmpd, compound; TE, thromboelastometry.

increased in the case of **38** that bears a 2-aminophenyl amide as ZBG. Further, we assessed whether **38** impairs the activity of fibrinolytic proteins. As shown in the [Supporting Information](#) (Figures S1–S3), compound **38** modified neither thrombin and tPA activity, nor plasmin generation. At this point, compounds that showed a good phenotypic response ($EC_{50} \leq 900$ nM), and compound **26** (in order to compare with our previously described lead compound CM-352), were advanced and evaluated in terms of cytotoxicity. Cell viability was determined in the healthy hepatic cell line THLE-2 after 72 h of incubation.²⁷ As shown in [Table 4](#) compounds **18**, **26**, and **27** exhibited low THLE-2 cytotoxicity ($LC_{50} > 10$ μM) leading to an adequate therapeutic window bigger than one log unit. Compounds **10**, **16**, **21**, and **38** showed some cytotoxicity effect, and their corresponding therapeutic windows are

between 0.5 and one log unit. However, **24** resulted much more cytotoxic with a LC_{50} of 2800 nM and a therapeutic window under 0.5 log units. These molecules showed a good solubility, in saline; but its permeability (passive diffusion, PAMPA) is variable (details in the [Supporting Information](#), S12, S13, and [Table S4](#)).

Finally, we decided to test the *in vivo* efficacy of some selected molecules using the hyperfibrinolytic tail bleeding mouse model test.²⁶ Bleeding time (defined as the interval between the initial transection and the visual cessation of hemorrhaging) was measured after administration of 0.5 mg/kg of tissue plasminogen activator (tPA). For this test, we selected compounds **18**, **26**, **27**, and **38**, molecules with an adequate therapeutic window (≥ 1 log unit). As shown in [Table 4](#), compound **26** did not reduce the bleeding time compared to saline-treated control; however, **18** was able to significantly reduce the bleeding time versus saline, although not versus the current standard of care (SoC), TXA. Better results were obtained with **27**; this molecule was able to significantly reduce the bleeding time versus TXA. Finally, **38** showed an outstanding *in vivo* efficacy, the best antifibrinolytic agent of these novel chemical series; its bleeding time was 3.6 min, thus significantly outperforming TXA, three times more efficacious at 300 times lower dose.

All these results presented above demonstrate that our compounds act as antifibrinolytics through an unknown MoA, MMP3- and MMP10-independent. We also assayed this compound **38** against two other MMPs (MMP7 and MMP14) that have been described to be involved in the regulation of the fibrinolytic system,²⁸ but **38** was inactive (details in the [Supporting Information](#), [Table S3](#)). Thus, our next aim is to decipher the underlying MoA leading to this phenotypic response and remarkable *in vivo* efficacy, and as first step, we should identify the primary target for these molecules.

To this end, we have considered a chemical proteomic strategy, activity-based protein profiling (ABPP), to identify the target associated with this phenotypic response in the native biological system.²⁹ Then, at this moment, we have already defined, synthesized, and characterized an ABPP-probe (also known as affinity-probe) to try to identify the target of these molecules leading to the reported antifibrinolytic response.

The synthesized affinity-probe, **39** ([Figure 2A](#), synthetic details described in the [Supporting Information](#)), possesses two critical components, in addition to its key chemical-features, to achieve its functional response, to perform its function: (i) a photoreactive group (UV irradiation-induced), benzophenone, for covalent labeling of the active site and (ii) a functional group (alkyne) ready for click-chemistry, thus enabling tagging and enrichment.³⁰ This compound was first tested for its inhibitory activity against MMP3 and MMP10, and as expected, it resulted inactive for both metalloproteinases. Compound **39** was also inactive against MMP7 and MMP14 (details in the [Supporting Information](#), [Table S3](#)). Then, its phenotypic activity was evaluated in the thromboelastometry assay obtaining a EC_{50} of 900 nM; thus, as required, **39** is a potent antifibrinolytic agent whose MoA is MMP independent ($IC_{50s} > 10$ μM).

Next step, not performed yet, involves (a) the incubation of the affinity-probe **39** with human whole blood, (b) irradiation to activate the photoreactive group and covalently link to its unknown target, (c) click chemistry conjugation with a reporter tag (biotin), (d) avidin enrichment, (e) on-beads trypsin digestion, and (f) LC–MS/MS analysis of protein fragments to

Table 4. Cytotoxicity and *in Vivo* Data of Selected Compounds

Cmpd	THLE-2 LC ₅₀ (nM) ^a	therapeutic window (log units)	bleeding time (min) at 1 mg/kg ^b	P-value vs saline ^c	P-value vs TXA ^c
10	3900	0.637			
16	7220	0.904			
18	16200	≥1.607	20.24 ± 3.83	0.05	0.28
21	3530	≥0.946			
24	2800	0.493			
26	>100000	>1.432	21.43 ± 3.80	0.14	0.19
27	43800	≥2.039	6.58 ± 1.68	<0.0001	0.05
38	3940	≥0.993	3.61 ± 1.21	<0.0001	<0.0001
saline			29.28 ± 0.4		
TXA ^d			14.2 ± 3.1		

^aAssay was performed in duplicate. ^bData reported as the mean value and SEM (standard error of the mean); *n* = 10. ^cData were evaluated using the Mann–Whitney U test. ^dTranexamic acid was tested at 300 mg/kg. Cmpd, compound; TXA, tranexamic acid.

Figure 2. (A) Chemical structure of compound 39. (B) Activity-based protein profiling approach to disclose the unknown target of these new series of antifibrinolytics.

identify the target (Figure 2B). These studies may lead to identify the unknown target of this new series of antifibrinolytic agents. In conclusion, by exploring alternative ZBGs we have discovered new chemical series that do not interact with metalloproteinases MMP3 and MMP10, in contrast with our previously described analogues,^{12,13} but behave *in vitro* and *in vivo* as potent antihemorrhagic agents.

Additional exploration around this novel chemical series led us to discovery a novel lead compound 38; this molecule was utilized as a pharmacological tool compound *in vivo* and showed a remarkable antihemorrhagic activity. In fact, 38 outperforms the current-standard-of-care (TXA). Finally, in order to decipher the underlying MoA leading to this phenotypic response and identify the primary target for these molecules, we have already identified an affinity-probe, 39, that is ready to achieve this aim.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.7b00549.

Schemes for preparation of final compounds, synthetic procedures, characterization of compounds, prep-HPLC, analytical HPLC, HRMS and LCMS methods, HPLC or LCMS traces of final compounds (PDF)

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Notes

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■ ABBREVIATIONS

ABPP, activity-based protein profiling; BBB, blood–brain barrier; MMP, metalloproteinase; MoA, mode-of-action; SoC, standard of care; TE, thromboelastometry; tPA, tissue plasminogen activator; TXA, tranexamic acid; ZBG, zinc binding group

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