Journal of Thrombosis and Haemostasis, **16**: 2035–2043 DOI: 10.1111/jth.142

BRIEF REPORT

The chaperone-like sodium phenylbutyrate improves factor IX intracellular trafficking and activity impaired by the frequent p.R294Q mutation

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To cite this article: Pignani S, Todaro A, Ferrarese M, Marchi S, Lombardi S, Balestra D, Pinton P, Bernardi F, Pinotti M, Branchini A. The chaperone-like sodium phenylbutyrate improves factor IX intracellular trafficking and activity impaired by the frequent p.R294Q mutation. *J Thromb Haemost* 2018; **16**: 2035–43.

Essentials

- Missense mutations often impair protein folding, and thus intracellular trafficking and secretion.
- Cellular models of severe type I hemophilia B were challenged with chaperone-like compounds.
- Sodium phenylbutyrate improved intracellular trafficking and secretion of the frequent p.R294Q.
- The increased coagulant activity levels (~3%) of p.R294Q would ameliorate the bleeding phenotype.

Summary. *Background:* Missense mutations often impair protein folding and intracellular processing, which can be improved by small compounds with chaperone-like activity. However, little has been done in coagulopathies, where even modest increases of functional levels could have therapeutic implications. *Objectives:* To rescue the expression of factor IX (FIX) variants affected by missense mutations associated with type I hemophilia B (HB) through chaperone-like compounds. *Methods:* Expression studies of recombinant (r)FIX variants and evaluation of secreted levels (ELISA), intracellular trafficking (immunofluorescence) and activity (coagulant assays) before and after treatment of cells with chaperone-like compounds. *Results:* As a model we chose the most frequent HB mutation (p.R294Q, ~100 patients), compared with other recurrent mutations associated with severe/moderate type I HB. Immunofluorescence studies revealed retention of rFIX variants in the endoplasmic reticulum and negligible localization in the Golgi, thus indicating impaired intracellular trafficking. Consistently, and in agreement with coagulation phenotypes in patients, all missense mutations resulted in impaired secretion (< 1% wild-type rFIX). Sodium phenylbutyrate (NaPBA) quantitatively improved trafficking to the Golgi and dose dependently promoted secretion (from 0.3 ± 0.1% to 1.5 ± 0.3%) only of the rFIX-294Q variant. Noticeably, this variant displayed a specific coagulant activity that was higher (~2.0 fold) than that of wild-type rFIX in all treatment conditions. Importantly, coagulant activity was concurrently increased to levels (3.0 ± 0.9%) that, if achieved in patients, would ameliorate the bleeding phenotype. *Conclusions:* Altogether, our data detail molecular mechanisms underlying type I HB and candidate NaPBA as affordable 'personalized' therapeutics for patients affected by the highly frequent p.R294Q mutation, and with reduced access to substitutive therapy.

Keywords: blood coagulation factor deficiencies; factor IX; hemophilia B; molecular medicine; missense mutation.

Introduction

Missense mutations are the main cause of human genetic disease and the most detrimental effect is exerted by amino acid changes impairing protein folding, thus leading to altered intracellular processing [1]. Protein folding is a complicated process that is assisted by chaperones, which are specialized molecules binding and stabilizing nascent polypeptides [2].

To counteract these detrimental effects, small molecules exhibiting chaperone-like activity have shown the ability

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Received: 7 March 2018
Manuscript handled by: R. Camire
Final decision: P.H. Reitsma, 1 July 2018

to rescue protein biosynthesis impaired by missense mutations [3–5]. Despite the therapeutic potential of these compounds, only very few attempts have been made for secreted proteins, in particular in the coagulation field, with a very limited number of mutations and molecules being challenged [6,7]. It is worth noting that even a modest increase from barely detectable (< 1%) to appreciable (3–5%) functional protein levels in plasma of coagulation factor-deficient patients is likely to ameliorate the clinical phenotype [8]. So far, small molecules have been extensively explored to induce ribosome readthrough and rescue nonsense mutations [9–12], another relatively frequent cause of coagulopathies [13,14].

Here, for the first time, we investigated chaperone-like compounds to rescue factor IX (FIX) expression impaired by missense mutations that are associated with proportionally low FIX activity and antigen levels, and thus type I hemophilia B (HB) [15]. This mutation type is by far the most frequent in HB, and we addressed a panel of *F9* missense mutations that includes the most represented in type I HB (p.R294Q). By extensive expression studies we detailed the defective intracellular processing of the selected FIX variants. Through a screening of chaperone-like compounds we identified sodium phenylbutyrate that, in a dose-dependent manner, appreciably improved intracellular trafficking and, most importantly, secretion of functional FIX impaired by the p.R294Q mutation.

Materials and methods

Creation of expression vectors

Expression vectors for recombinant FIX (rFIX) variants were produced by mutagenesis of human *F9* cDNA, cloned into pCDNA3, as described [16]. The forward oligonucleotides 5′GATGACATTAATTCCTGTGAATG TTGGTGTCCC3′ (pFIX-115C), 5′CCTGTACTGAGGG ATGTCGACTTGCAGAAAAC3′ (pFIX-161C), 5′CAGA GCAAAAGCAAAATGTGATTCG3′ (pFIX-294Q) and 5′CGAATTATTCCTCACCACAACTGCAATGCAGCT ATTAATAAG3′ (pFIX-305C) were used. Reverse oligon ucleotides were complementary to the forward ones. All plasmids have been validated by sequencing.

Cell transfection and treatment with compounds

Expression studies were performed in human embryonic kidney 293 (HEK293) cells as described [11,17]; 4-phenylbutyrate, sodium phenylbutyrate (NaPBA), N-acetylcysteine and ambroxol were from Sigma-Aldrich (St. Louis, MO, USA); VX-809 was from Selleckchem (Houston, TX, USA). Stable clones were treated with chaperone-like compounds in 24-well plates, and media and cell lysates collected 48 h later.

Evaluation of rFIX protein levels

Secreted rFIX variants were evaluated by ELISA through polyclonal antibodies (Affinity Biologicals, Ancaster, ON, Canada). Known concentrations of purified rFIX were used as reference. The rFIX in cell lysates was revealed by western blotting [12].

Immunofluorescence studies

Stable clones, seeded on a 12-mm glass slide within 24-well plates and maintained for 24 h in vitamin K-supplemented medium, were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Non-specific binding sites were blocked with PBS-5% milk-0.1% Triton X-100 (blocking buffer). Polyclonal goat anti-human FIX (1:200; Affinity Biologicals), polyclonal rabbit anti-PDI (protein disulfide-isomerase) (1:200; Abcam, Cambridge, UK) and monoclonal mouse anti-GM130 (1:100; Becton Dickinson, Franklin Lakes, NJ, USA) antibodies were diluted in blocking buffer and incubated overnight at 4 °C. Detection was performed by Alexa Fluor-conjugated donkey anti-goat (594 nm; FIX), anti-rabbit (488 nm; PDI) and anti-mouse (647 nm; GM130) antibodies (Thermo Fisher Scientific, Waltham, MA, USA) incubated for 1 h at room temperature. Images were acquired on the Zeiss LSM510 confocal microscope [18].

Coagulant activity of recombinant variants

Coagulant activity of rFIX variants was assessed by activated partial thromboplastin time (APTT)-based assays as previously described [11,12]. Coagulation times from a standard curve with serial dilutions of stably expressed wild-type rFIX (rFIX-wt) were used as reference.

Data analysis

Coagulant assays were analyzed by a two-phase decay non-linear fit of coagulation times with GraphPad Prism 5 (San Diego, CA, USA) [19]. Immunofluorescence images were analyzed by ImageJ software (https://imagej.net) [20]. Statistical differences were analyzed by *t*-test.

Results and discussion

Missense mutations, representing > 55% of *F9* gene alterations listed in reports [13,21] and databases (www.factorix.org) [14], are associated with heterogeneous phenotypes, with severe forms being caused by FIX coagulant levels below 1% of normal [15]. We focused on the p.R294Q, the most frequent *F9* missense mutation ($n = 97$) associated with severe/moderate type I HB (Fig. 1A, B) [22], and thus a candidate to affect intracellular processing. As a comparison, we included a panel of

A

Patients (*n*)	Mutation (HGVS)	Mature FIX numbering	Nucleotide change	CpG	Exon	Protein Domain
97	p.R294Q	248	c.881G>A	Y	8	Catalytic
9	p.Y115C	69	c.344A>G	N	4	EGF1
9	p.Y305C	259	c.915A>G	N	8	Catalytic
5	p.Y161C	115	c.482A>G	N	5	EGF2

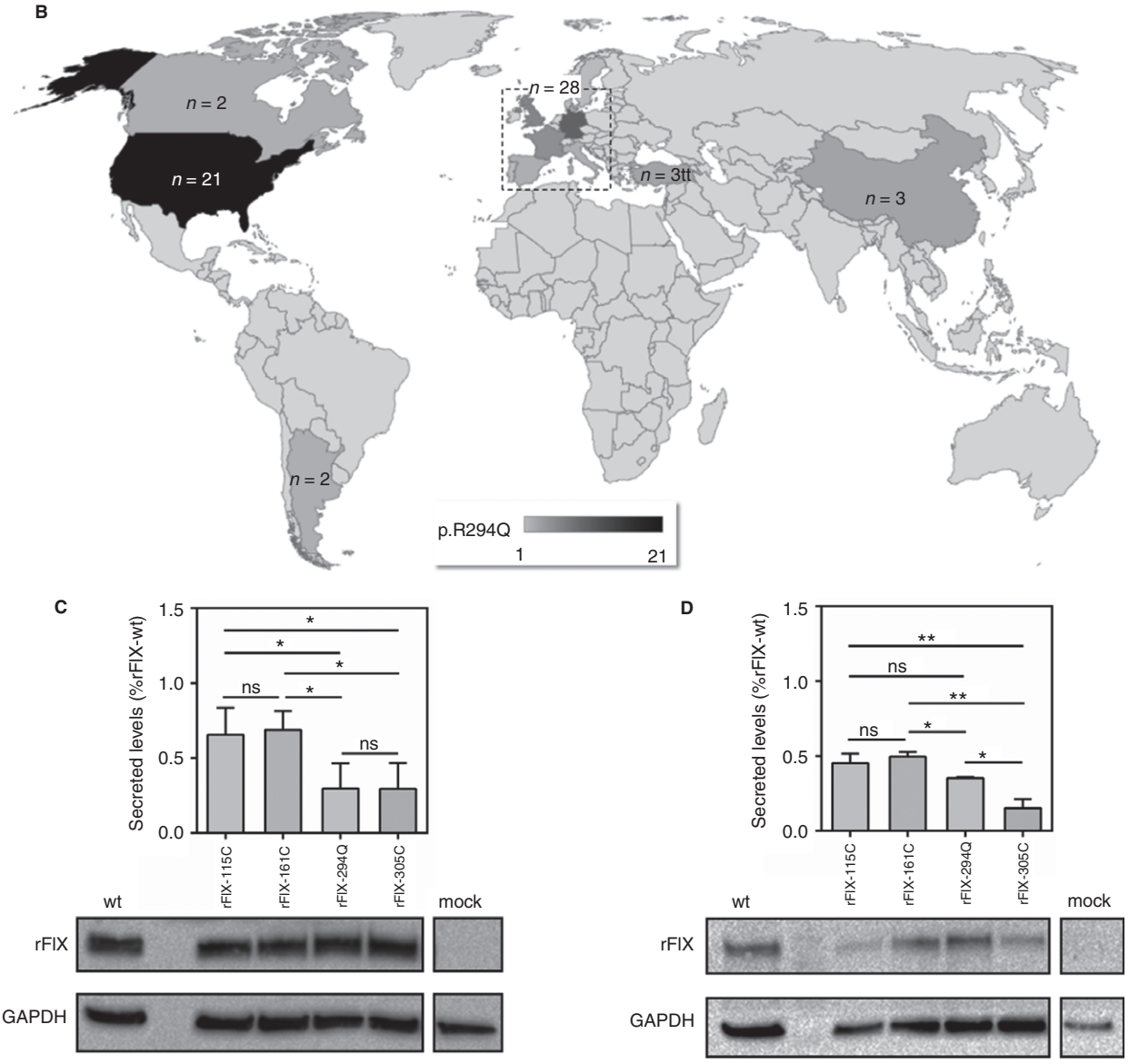


Fig 1. *F9* mutations and evaluation of secretion efficiency of rFIX variants. (A) Selected *F9* missense mutations reported in the FIX variant database (www.factorix.org). HGVS, Human Genome Variation Society; EGF, epidermal growth factor. (B) Representation of the p.R294Q mutation frequency distribution. Numbers of patients from published reports are shown. (C) Transient expression studies were conducted in HEK293 cells. Protein levels of rFIX variants were evaluated in conditioned media by ELISA (upper panel) and in cell lysates by western blotting (lower panel, with GAPDH as loading control). (D) Transient expression of rFIX missense variants in Chinese Hamster Ovary cells. Upper and lower panels are as in C. Results in C and D are reported as % of rFIX-wt (mean ± standard deviation from three independent experiments). ns, not significant; $*P < 0.05$; $**P < 0.005$; wt, wild-type; Mock, cells transfected with a gutted vector; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

recurrent mutations associated with severe HB, namely the p.Y115C ($n = 9$), p.Y161C ($n = 5$) and p.Y305C ($n = 9$) substitutions.

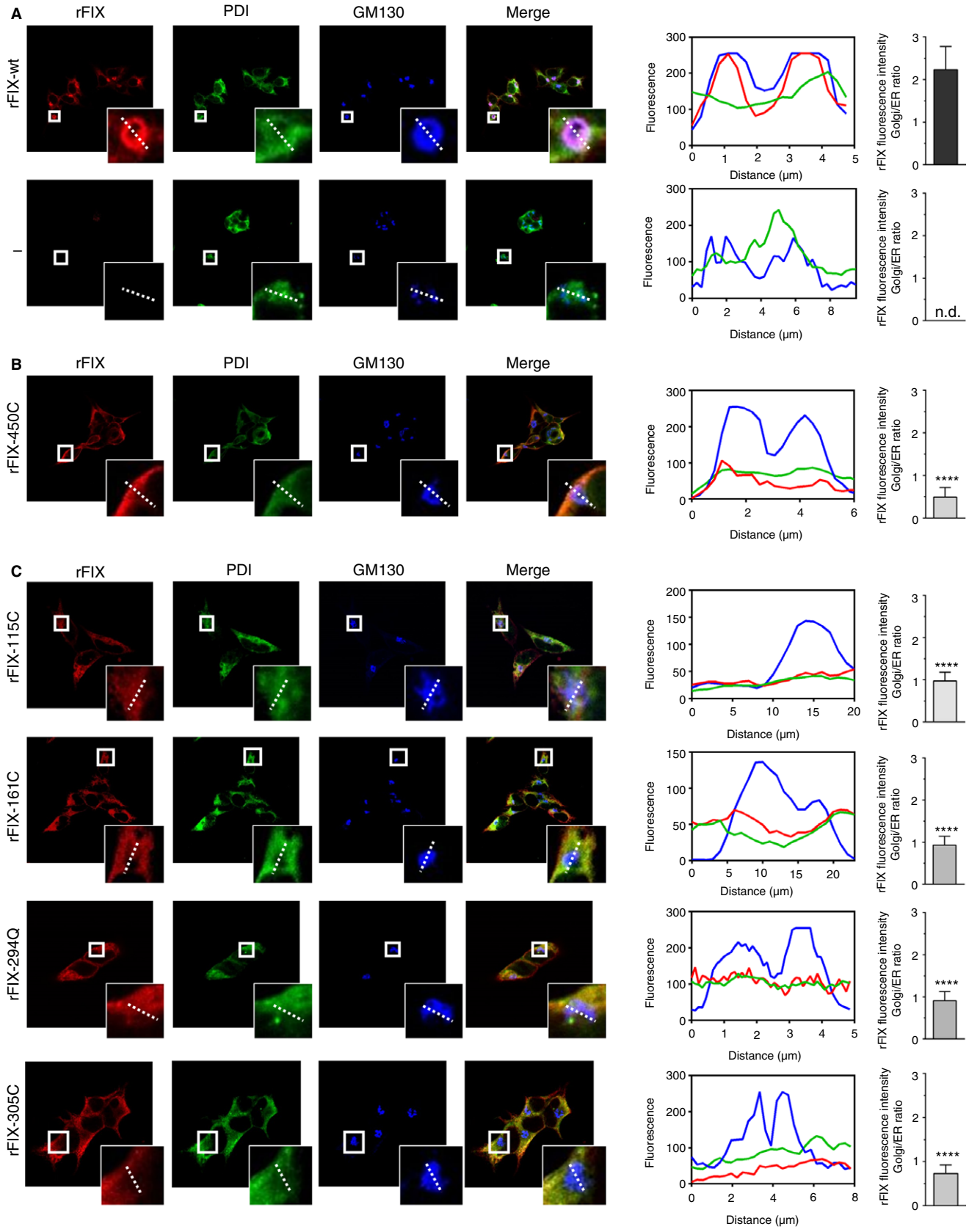
Mutations were characterized by expression of rFIX variants in HEK293 cells, known to properly synthesize FIX with its complex post-translational modifications [23], and evaluation at the extracellular and intracellular level. To evaluate secretion efficiency of rFIX variants, we initially performed transient expression studies. The ELISA in conditioned media revealed extremely reduced (from ~0.3% to ~0.7% of rFIX-wt) secreted levels for all variants (Fig. 1C, upper panel) that displayed a size comparable to that of rFIX-wt in cell lysates (Fig. 1C, lower panel). To corroborate these findings, we also expressed the rFIX variants in Chinese Hamster Ovary cells, another well-established cell line to characterize coagulation factor variants [24]. In this experimental system, rFIX-wt was secreted in medium at levels (424 ± 170 ng mL^{-1}) comparable to those measured from HEK293 cells (441 ± 25 ng mL^{-1}), and rFIX missense variants confirmed a very inefficient secretion (from ~0.2% to ~0.5% of rFIX-wt) (Fig. 1D). These data indicated that the selected missense mutations impair FIX secretion, thus demonstrating their association with severe type I deficiency and providing us with a HEK293-based experimental system to investigate rescue by chaperone-like compounds.

To overcome the intrinsic variability related to transient expression, which might confound the evaluation of treatment effects, we selected stable clones. As in the previous system, we detected very low secreted rFIX levels (< 1% of rFIX-wt) for all missense variants, which validated the experimental model. Immunostaining of rFIX, and of the endoplasmic reticulum (ER) or Golgi markers, was exploited to trace intracellular rFIX trafficking through the secretory pathway [23]. Confocal microscopy followed by analysis of the three fluorescence signals clearly localized rFIX-wt within the ER and particularly in the Golgi (Fig. 2A), consistent with proper trafficking towards secretion. Conversely, the rFIX variant affected by the p.Y450C mutation, previously demonstrated to cause severe type I HB [16] and here chosen as additional control, was strongly detectable in the ER but barely in the Golgi, as indicated by the negligible qualitative and quantitative co-localization of the red (rFIX) and blue (Golgi) fluorescence signals (Fig. 2B). The rFIX-294Q, as well as the other variants, showed a fluorescence pattern comparable to that of rFIX-450C, thus indicating impaired intracellular trafficking and supporting rFIX retention in the ER (Fig. 2C). The data, demonstrating for the first time the pathogenic mechanisms of these mutations, were consistent with the remarkably reduced rFIX secreted levels and the associated type I HB forms.

Amino acid changes can have pleiotropic effects and, besides secretion, they also frequently impair function, a particularly critical issue for proteins with enzymatic activity. As a matter of fact, the combination of reduced secretion and functional impairment is the determinant of coagulation phenotype in patients [16,24]. In addition, functional impairment would make unsuccessful therapeutic attempts based on promoting intracellular protein processing and secretion, as in the case of chaperone-like compounds. We therefore evaluated coagulant properties of rFIX missense variants in FIX-deficient plasma systems through APTT-based assays. As shown in Fig. 3(A), the specific coagulant activity, referred to as the ratio between coagulant activity and secreted protein levels, was undetectable for the rFIX-115C and rFIX-161C variants and barely detectable for the rFIX-305C (0.04 ± 0.01). Intriguingly, the rFIX-294Q mutant displayed a specific coagulant activity that was higher (~2.0 fold) than that of rFIX-wt. All these elements make the rFIX-294Q variant a promising candidate to explore chaperone-like compounds.

Screening for rescue demonstrated that the 4-phenylbutyrate, VX-809, N-acetylcysteine and ambroxol compounds were ineffective at any concentration tested (data not shown). Intriguingly, NaPBA, which did not affect the rFIX-wt expression (Fig. 4A, left panel), appreciably improved secretion of the rFIX-294Q variant with a dose-dependent effect (Fig. 4A, left/middle panels). In particular, as compared with rFIX-wt, secreted levels for the rFIX-294Q variant increased from $0.3 \pm 0.1\%$ without treatment to $1.5 \pm 0.3\%$ at the highest NaPBA concentration tested (2 mM), as revealed by two different polyclonal ELISA settings. Consistently, investigations at the

Fig. 2. Evaluation of intracellular trafficking of rFIX variants. Immunofluorescence studies were conducted in stably transfected HEK293 cells. Stable clones were isolated (n°48 for each rFIX variant) and seeded in multi-well plates in the presence of G418 (500 μg mL^{-1}) as selection agent and 5 μg mL^{-1} vitamin K. Investigation by polyclonal ELISA revealed very low secreted rFIX levels for all variants. Fluorescently labelled secondary antibodies were used to visualize rFIX (red signal) as well as PDI (ER, green) or GM130 (Golgi, blue) protein markers. Image magnifications (white square) for each channel as well as for the integration of the three signals (merge) are shown. The HEK293 clones expressing rFIX-wt (A) and rFIX-450C (B) proteins were used as controls for normal or defective rFIX expression of the investigated missense variants (C), respectively. HEK293 cells alone were used as negative control (−). Selected regions (image magnifications, white dotted lines) were analyzed for co-localization of the rFIX signal (red) relative to the ER (PDI, green) and Golgi (GM130, blue) as a function of the distance (μm) of the analyzed region (three-color plots). Histograms report the ratio of the distribution of the rFIX fluorescence in the Golgi and ER that was estimated from at least 10 cells for each variant and is expressed as mean ± standard deviation (****$P < 0.0001$; n.d., not detectable). rFIX, recombinant factor IX; PDI, protein disulfide-isomerase; ER, endoplasmic reticulum; GM130, Golgi matrix protein 130.



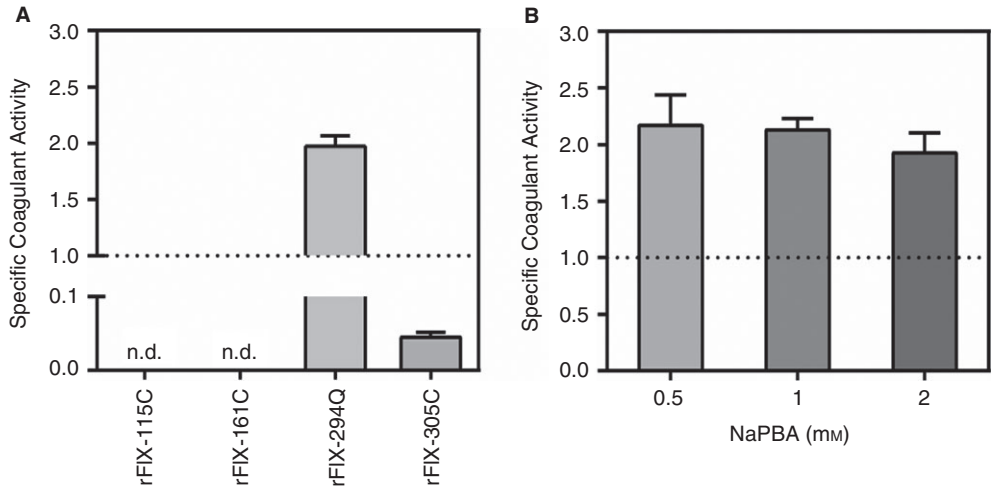


Fig. 3. Specific coagulant activity of rFIX missense variants. (A) Specific coagulant activity (activity/antigen ratio) of rFIX variants secreted from stable HEK293 clones. Two dilutions (1:2, 1:4) in mock medium were assessed. Coagulation was triggered in FIX-depleted plasma (HemosIL, Instrumentation Laboratory, Lexington, MA, USA), supplemented with rFIX-containing medium, by addition of the contact activator SynthASil (HemosIL) and $CaCl_2$. Coagulation times were measured on an ACLTOP700 instrument (Instrumentation Laboratory). The estimated CV% of the assay was 8.7%. n.d., not detectable. (B) Specific coagulant activity of the rFIX-294Q variant upon treatment with increasing concentrations of NaPBA. The dotted line represents specific activity of rFIX-wt. Results are reported as mean ± standard deviation from three independent experiments. As negative control we measured coagulation times in medium from untransfected HEK293 cells untreated or treated with NaPBA (0.5, 1 and 2 mM), which provided overlapping results. NaPBA, sodium phenylbutyrate.

intracellular level by immunofluorescence revealed that NaPBA appreciably improved rFIX-294Q trafficking to the Golgi compartment, as witnessed by the qualitative distribution of rFIX and GM130 fluorescence signals. Indeed, at variance with untreated rFIX-294Q-expressing cells, the overlapping of the two signals was clearly detectable after treatment with NaPBA and the co-localization of rFIX fluorescence intensity was quantitatively improved (Fig. 4B, histograms). Noticeably, improvement of rFIX-294Q secretion was paralleled by an increase in coagulant activity in medium from NaPBA-treated cells, as indicated by the appreciable, and dose-dependent, effect on coagulation times (Fig. 4A, right panel). Specifically, treatment resulted in a shortening of coagulation times from 80 ± 0.1 s in untreated conditions to 76 ± 4.7, 68 ± 7 and 62 ± 3 s upon treatment with 0.5, 1 and 2 mM NaPBA, respectively. As compared with rFIX-wt activity, this corresponded to an increase in coagulant activity levels from 0.5 ± 0.04% to 3.0 ± 0.9% at the highest dose (2 mM). Interestingly, for all treatment conditions, the rFIX-294Q variant showed an increased specific activity (2.2 ± 0.3, 2.1 ± 0.1 and 1.9 ± 0.2, respectively) in comparison with rFIX-wt (Fig. 3B), a feature that magnifies the functional impact of the NaPBA-mediated rescue. The same rescue profile was observed in an independent rFIX-294Q-expressing clone.

Structure-function studies indicated that arginine 294 maps in a region involved in the interaction with activated FVIII [25]. Whereas previous investigations did not permit clear definition of the specific activity of the p.R294Q variant [22,26,27], the low protein levels achieved in our recombinant system do not favor a proper comparison by a fine biochemical characterization of the variant. It is worth noting that the 'gain-of-function' feature is also suggested by inspection of the FIX variant database (www.factorix.org) that reports several patients with FIX activity levels slightly exceeding antigen levels, and a large proportion of patients with a moderate bleeding phenotype. This finding resembled the paradigmatic example of the p.R384X nonsense mutation upon drug-induced readthrough, which was strongly favored by the increased specific activity of the resulting missense variant [11]. Overall, these data demonstrate that NaPBA appreciably counteracts the detrimental effects of the p.R294Q mutation and improves rFIX intracellular trafficking and secretion of a functional FIX protein.

Altogether, our data detail molecular mechanisms underlying type I HB, characterized by defective intracellular trafficking and ER retention of the rFIX missense variants. For the first time we explored the functional impact of a panel of chaperone-like compounds in HB. Through complementary approaches in cellular models we provided experimental evidence, at the intracellular and secreted protein levels, on the ability of NaPBA to rescue, in a dose-dependent manner, FIX biosynthesis and function impaired by the *F9* p.R294Q, the most frequent type I HB mutation. Intriguingly, the functional impact of NaPBA-mediated rescue on the p.R294Q change was magnified by the improved specific activity of the secreted molecule. These features led to a coagulant activity (~3%) that, if achieved in HB patients, would favor the transition from a severe/moderate to a moderate/mild clinical picture and potentially alleviate the bleeding tendency. Taking into account the p.R294Q

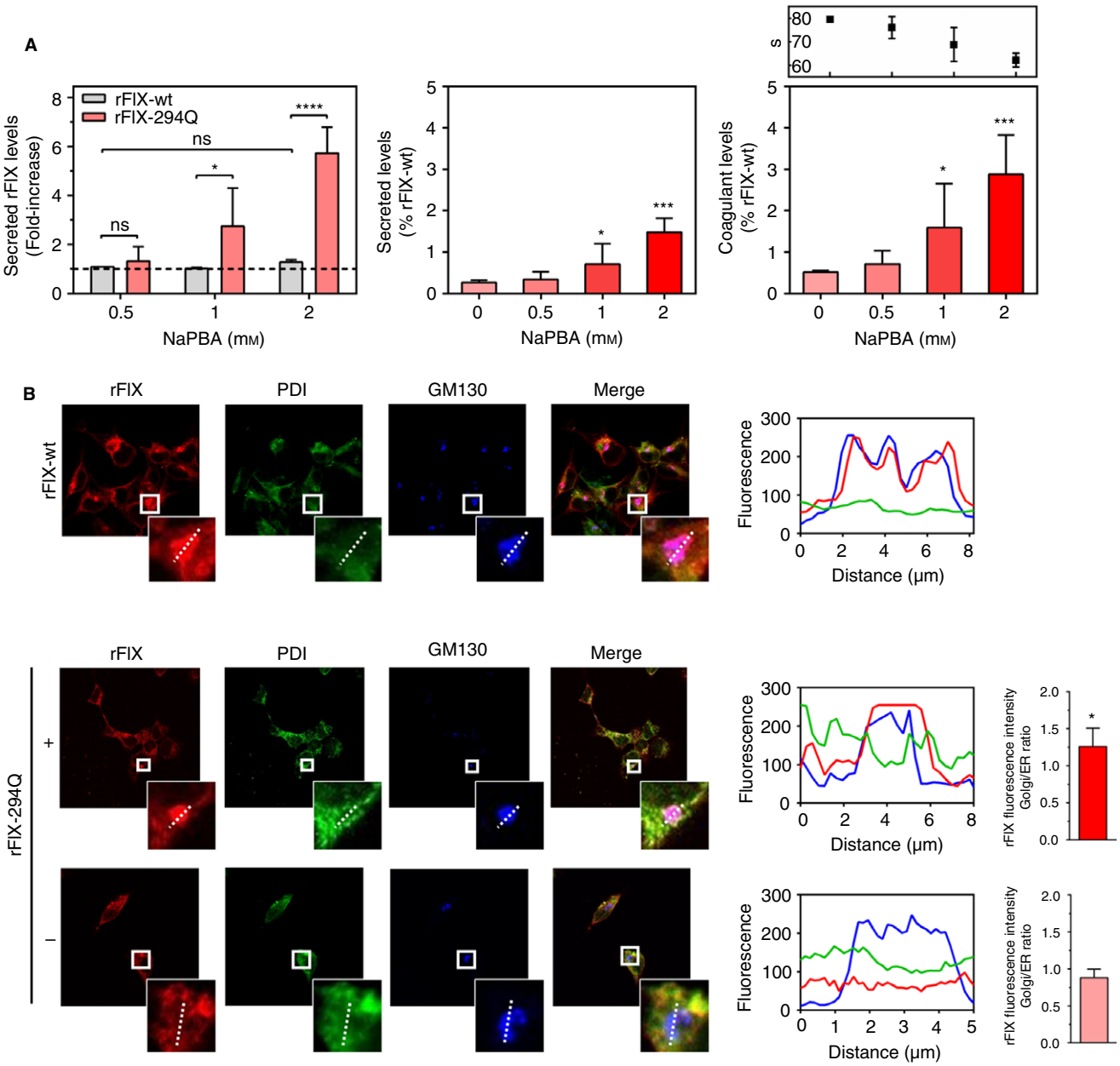


Fig. 4. Evaluation of NaPBA-mediated rescue of the rFIX-294Q missense variant. (A) Treatment of stable clones expressing rFIX-wt or rFIX-294Q (left panel) and relative increase of rFIX-294Q secreted (middle panel) and coagulant activity (right panel) levels. Shortening of coagulation times (sec, seconds), corresponding to each NaPBA concentration, is indicated on top of the right panel. Results are shown as fold-increase (left panel) of rFIX secreted levels as compared with those from untreated cells (set to 1 and indicated by the dotted line) and as % of rFIX-wt (middle and right panels) from three independent experiments (mean ± standard deviation). ns, not significant; $*P < 0.05$; $***P \leq 0.0005$; $****P < 0.0001$. (B) Localization of the rFIX-294Q variant in the absence (−) or in the presence (+) of 2 mM NaPBA. Co-localization of the rFIX signal (red) relative to the ER (PDI, green) and Golgi (GM130, blue) as well as of rFIX distribution in the Golgi and ER (results are reported by histograms as mean ± standard deviation; $*P < 0.05$) was analyzed as in Fig. 2. NaPBA, sodium phenylbutyrate; ER, endoplasmic reticulum; GM130, Golgi matrix protein 130; PDI, protein disulfide-isomerase.

frequency and its responsiveness to NaPBA that, albeit for different purposes [28], is approved for use in humans and can be orally administered, these data make NaPBA a candidate as an affordable 'personalized' therapeutics, particularly in countries or settings with poor access to standard therapies. It is worth noting that the number of patients affected by this mutation (Fig. 1B), increased both by recurrence at a CpG dinucleotide and potentially by founder effects [29,30], is very likely to be underestimated. Our data also suggest that rescue at the functional

level of severe FIX mutations is not a frequent finding, and requires a systematic analysis to find suitable candidates. We propose to extend this expression platform to the numerous but individually-rare missense mutations leading to severe type I hemophilia to select those that are responsive to chaperone-like compounds, which would lay the foundation for further studies in animal models to assess the therapeutic potential.

Addendum

S. Pignani performed immunofluorescence studies and evaluated rFIX protein levels as well as coagulant activity in APTT-based assays. A. Todaro performed experiments with chaperone-like compounds. M. Ferrarese created recombinant plasmids and performed transient expression studies. S. Marchi provided support for immunofluorescence studies and performed analysis of acquired images by confocal microscope. S. Lombardi and D. Balestra created and validated stable clones expressing rFIX variants. P. Pinton analyzed immunofluorescence data and carefully revised the manuscript. A. Branchini and M. Pinotti conceived the study and designed the research. A. Branchini, M. Pinotti and F. Bernardi analyzed and interpreted data and wrote the manuscript. All authors revised and approved the final version of the manuscript.

Acknowledgements

This study was financially supported by an unconditioned research grant from Pfizer (EuroAspire project WI199905 to A. Branchini.). Pfizer had no role in study design, data analysis and interpretation of results. The authors would like to thank Dr R. Mari for technical assistance in performing coagulation assays.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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