

Pharmacological versus genetic inhibition of heme oxygenase-1 – the comparison of metalloporphyrins, shRNA and CRISPR/Cas9 system

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Inhibition of heme oxygenase-1 (HO-1, encoded by *HMOX1*), a cytoprotective, anti-apoptotic and anti-inflammatory enzyme, may serve as a valuable therapy in various pathophysiological processes, including tumorigenesis. We compared the effect of chemical inhibitors – metalloporphyrins, with genetic tools – shRNA and CRISPR/Cas9 systems, to knock-down (KD)/knock-out (KO) HO-1 expression/activity. 293T cells were incubated with metalloporphyrins, tin and zinc protoporphyrins (SnPPIX and ZnPPIX, respectively) or were either transduced with lentiviral vectors encoding different shRNA sequences against HO-1 or were modified by CRISPR/Cas9 system targeting *HMOX1*. Metalloporphyrins decreased HO activity but concomitantly strongly induced HO-1 mRNA and protein in 293T cells. On the other hand, only slight basal HO-1 inhibition in shRNA KD 293T cell lines was confirmed on mRNA and protein level with no significant effect on enzyme activity. Nevertheless, silencing effect was much stronger when CRISPR/Cas9-mediated knock-out was performed. Most of the clones harboring mutations within *HMOX1* locus did not express HO-1 protein and failed to increase bilirubin concentration after hemin stimulation. Furthermore, CRISPR/Cas9-mediated HO-1 depletion decreased 293T viability, growth, clonogenic potential and increased sensitivity to H₂O₂ treatment. In summary, we have shown that not all technologies can be used for inhibition of HO activity *in vitro* with the same efficiency. In our hands, the most potent and comprehensible results can be obtained using genetic tools, especially CRISPR/Cas9 approach.

Key words: CRISPR/Cas9, shRNA, inhibitors, heme oxygenase-1, HO-1, off-target

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Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; HO-1, heme oxygenase-1; KD, knock-down; SnPPIX, tin protoporphyrin; ZnPPIX, zinc protoporphyrin

INTRODUCTION

Heme oxygenase-1 (HO-1, encoded by *HMOX1*), first characterized in 1968 (Tenhunen *et al.*, 1968), is an enzyme responsible for the degradation of pro-oxidant heme, which leads to the release of carbon monoxide

(CO), ferrous ion (Fe²⁺), and biliverdin. Besides its antioxidant function (Stocker, 1990), HO-1 is considered as a regulator of cellular homeostasis, a modulator of inflammatory response and angiogenesis. Unfortunately, all of potentially beneficial properties of HO-1 can contribute to pathophysiological processes, such as diabetes, hypertension or tumorigenesis (Deshane *et al.*, 2005). Indeed, a growing body of evidence suggests an important role of HO-1 in various types of cancer (Loboda *et al.*, 2015). Therefore, inhibition of HO-1 can potentially serve as an anticancer therapy.

Several approaches for HO-1 silencing are used in experimental settings; however, due to the off-target effects, their experimental and possibly therapeutic application, at least in some cases, is strongly limited. Pharmacological agents, like commonly used metalloporphyrins might, apart from targeting HO, modulate the activity of nitric oxide synthase (NOS), soluble guanylate cyclase (sGC) and cytochrome P450 (Loboda *et al.*, 2015). The most commonly used genetic tools for specific HO-1 inhibition are small interfering RNA (siRNA) or short hairpin RNA (shRNA) that bind to a complementary sequence in targeted mRNA and predominantly induce its cleavage (Rao *et al.*, 2009). *In vitro*, decreased levels of HO-1 achieved by RNAi resulted in increase in apoptosis of lung cancer cells (Kim *et al.*, 2008) and augmented sensitivity to anticancer therapy in pancreatic cancer cells (Berberat *et al.*, 2005). Moreover, siRNA-mediated HO-1 downregulation led to diminished growth of tumours *in vivo* in the orthotopic model of hepatocellular carcinoma (Sass *et al.*, 2008). Although the RNAi approach is very useful in experimental settings, its potential therapeutic application remains limited. Major obstacles include inefficient delivery of the genetic material, variable knock-down efficiencies, poor stability due to the degradation by RNases present in body fluids and, in some cases, the possible induction of immune response (Reynolds *et al.*, 2006; Aagaard & Rossi, 2007). Additionally, siRNA and shRNA sequences can exhibit off-target effects by inhibiting undesired genes (Qiu *et al.*, 2005).

CRISPR/Cas9 system was adopted from bacterial immune defense system against foreign DNA and modified for the purpose of genome engineering. In this approach, Cas9 promotes genome editing by stimulating a double-strand breaks (DSB) formation at a target genomic locus. DSB can be repaired either by non-homologous end joining (NHEJ) or homology-directed repair (HDR). DSB repair by NHEJ often results in small insertions or

deletions (indels) and, if DSB occurred within coding region of a gene, indel mutations can change the mRNA reading frame leading to the introduction of premature stop codon and, as a consequence, gene knock-out (Hendriks *et al.*, 2016). CRISPR/Cas9 has provided an easy and efficient genome editing tool in cultured human cells (Cho *et al.*, 2013; Cong *et al.*, 2013; Mali *et al.*, 2013), *Drosophila* (Gratz *et al.*, 2013), zebrafish (Hwang *et al.*, 2013), or plants (Xie & Yang 2013) and currently is a widely used approach for generation of knock-out animal models, due to its simplicity, efficiency, and specificity (Housden *et al.*, 2017). As a new and still developing method, its advantages, drawbacks, and applications still need to be determined.

The aim of the present study was to compare the effectiveness of both pharmacological and genetic inhibition of HO-1 in a model cell line, human embryonic kidney 293T cells, and to identify the possible benefits and limitations of the used methods.

MATERIALS AND METHODS

Cell culture. Human embryonic kidney 293T cells (kindly obtained from dr Maciej Wiznerowicz from Wielkopolskie Centrum Onkologii, Poznan, Polska) were cultured under standard conditions (37°C, 5% CO₂) in high glucose (4.5 g/L) Dulbecco's Modified Eagle's medium – DMEM (Lonza, Basel, Switzerland), supplemented with 10% fetal bovine serum (FBS, Biowest, Nuaille, France/EURx, Gdansk, Polska) and antibiotics: 100 U/mL penicillin and 100 µg/mL streptomycin (Lonza, Basel, Switzerland).

Cells stimulation. Cells growing in 96-well plates (3000 or 5000/well), 48-well plates (50000/well), 24-well plates (200000/well), 6-well plates (650000/well) or 10 cm dishes (2.0 × 10⁶/dish) were stimulated for cell viability or proliferation, immunofluorescence, RNA isolation, protein analysis and HO activity, respectively. SnPPIX and ZnPPIX (Frontier Scientific, Logan, UT, USA, dissolved in DMSO) were used at 0.5–25 µM for 6 h or 24 h, whereas hemin (Calbiochem, San Diego, CA, USA), was dissolved in phosphate buffer with 10 mM NaOH and added at 50 µM for 6 h or at 10 µM for 24 h. Hydrogen peroxide (H₂O₂) at 0.05–0.5 mM concentration was used for 24 h stimulation. The control cells received the appropriate solvent.

Production of lentiviral vectors encoding shRNA sequences against HMOX1. Four 29-mer shRNA constructs (shHO-1 A-D) against human *HMOX1* and one 29-mer non-targeting shRNA (scrambled shRNA) in pGFP-C-shLenti vectors were purchased from OriGene (Rockville, MD, USA). 293T cells were transfected with plasmids of interest and two helper plasmids: pMD2.G and psPAX2as described previously (Szulc *et al.*, 2006) using polyethylenimine – PEI (Polysciences, Inc., Warrington, PA, USA) as a transfection reagent. The plasmids were a gift from Didier Trono (Addgene plasmids #12259 [pMD2.G] and #12260 [psPAX2]). Transfection efficiency was monitored under fluorescent microscope by the assessment of GFP positive cells. 48 h and 72 h after transfection, media containing lentiviral vectors were collected and pooled. The vector titers were determined by transduction of 293T cells with lentiviral vectors and subsequent analysis of the fraction of GFP positive cells using flow cytometer (BD Fortessa).

Stable transduction with lentiviral vectors. 293T cells were transduced with lentiviral vectors encoding either scrambled shRNA (control cells) or one of four

shRNAs against *HMOX1* (shHO-1 A-D) in the presence of 5 µg/mL polybrene (Sigma-Aldrich, St. Louis, MO, USA). Multiplicity of infection (MOI) of 20 was used and after 72 h the GFP positive cells were sorted using MoFlo XDP cell sorter (Beckman-Coulter).

Knock-out 293T generation. For the generation of HO-1 deficient 293T cells pX330-Pac-Cer plasmid that encode sgRNA targeting human *HMOX1* locus or an empty vector (control cells) has been used as described previously (Czarnek & Bereta, 2017). Cell clones containing mutations in *HMOX1* locus (assessed by CELI mismatch detection assay) were used for further analyses.

Colony formation assay. 1000 cells were seeded on 12-well plates and grown for 7 days. Afterwards cells were fixed with cold (–20°C), 100% methanol for 20 minutes on ice and stained with 0.05% (w/v) crystal violet (BioShop, Burlington, Ontario, Canada) in 20% methanol for 20 minutes at room temperature. Crystal violet was then completely removed and pictures of the plates were taken using Fusion FX5 XT camera (Vilber).

MTT viability assay. To assess viability of cells, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, St. Louis, MO, USA) assay was used. After 24 h stimulation with H₂O₂, cells were incubated with 1 mg/mL MTT in medium for 2 h at 37°C. Then, formazan crystals were dissolved in 100 µL/well of lysis buffer consisting of 0.6% (v/v) acetic acid and 10% (w/v) SDS in DMSO and absorbance at 570 nm with the reference value of 690 nm was measured using Infinite M200 microplate reader (Tecan).

Cell proliferation. To determine cell growth over the time, CyQUANT Cell Proliferation Assay (Thermo Fisher Scientific, Waltham MA, USA) was performed according to the manufacturer's instructions.

Heme oxygenase activity assay. Heme oxygenase activity was determined in cell lysates according to the method previously described (Foresti *et al.*, 1997) with slight modification regarding the source of biliverdin reductase (BVR). BVR was produced in BL21(DE3) RIL competent bacteria after transformation using pET24a+::rBVR plasmid with kanamycin resistance. Bacteria were cultured in LB with 30 µg/mL kanamycin at 37°C until optical density (OD₆₀₀) reached values between 0.6–0.8. Induction of rBVR expression was carried out by addition of 0.1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 5 h at 30°C. Bacteria pellet was dissolved in buffer containing 20 mM imidazol, 300 mM NaCl and 20 mM Tris/HCl, pH 7.4 in ratio: 1 g of the bacteria to 5 mL of the buffer and lysed by sonification. After centrifugation (7500 rpm, 5 minutes), purification of the rBVR was performed by loading the supernatant on a column packed with Ni Sepharose HighPerformance resin. Elution of the rBVR was carried out by using buffer with 500 mM imidazol, 300 mM NaCl and 20 mM Tris/HCl pH 7.4. After elution protein was dialyzed for 18 h at 4°C against 300 mM NaCl and 20 mM Tris/HCl pH 7.4 buffer. Subsequently protein was condensed to about 10 mg/mL concentration, glycerol was added to final 50% concentration and rBVR was frozen at –20°C. 25 µg/ml BVR was used for HO activity assay.

In brief, after 6 h stimulation with 50 µM hemin cells were scraped in 100 mM phosphate buffer with 2 mM MgCl₂. After three cycles of freeze (in liquid nitrogen) and thaw (at 37°C) cell lysate was added to a reaction mixture containing NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase (Sigma-Aldrich, St. Louis, MO, USA), BVR and the substrate hemin. After 1 h incubation of the above mixture in the dark at 37°C the reaction was terminated by the addition of chloro-

form (POCH, Gliwice, Polska). Followed vigorous vortexing (extraction) and centrifugation (phase separation), the concentration of extracted bilirubin was measured by the difference in absorbance between 464 nm and 530 nm ($\epsilon = 40 \text{ mM}^{-1} \text{ cm}^{-1}$) and calculated as picomole of bilirubin formed per milligram protein per hour.

Quantitative RT-PCR. RNA was isolated from cultured cells using Fenzol (A&A Biotechnology, Gdynia, Polska) with modified Chomczynski and Sacchi method (Chomczynski & Sacchi, 1987). RNA concentration and purity were determined using NanoDrop 1000 (Thermo Fisher Scientific, Waltham MA, USA). Reverse transcription was performed with SuperScript polymerase (Invitrogen, Carlsbad, CA, USA) according to instructions supplied by the manufacturer. Obtained cDNA was used as a template for quantitative real-time PCR with SybrGreen Mix (Sigma-Aldrich, St. Louis, MO, USA) and specific primers (*HMOX1* F: 5'-TTCTTCACCTTC-CCCAACATT-3'; R: 5'-CAGCTCCTGCAACTCCT-CAAA-3'; *HMOX2*: F: 5'-GATCGTGGAGGAGGC-CAACA-3'; R: 5'-TAGAAAGGGCATTTACGCAT-3'; *EEF2* F: 5'-GAGATCCAGTGTCCAGAGCAG-3'; R: 5'-CTCGTTGACGGCAGATAGG-3'). *EEF2* (encoding eukaryotic translation elongation factor 2, EF-2) was used for gene expression normalization. The reaction was performed using a StepOnePlus™ Real-time PCR Systems (Applied Biosystems).

Western blot. Cultured cells were lysed with RIPA buffer and 25–30 μg of protein lysates were loaded on 12% SDS-PAGE gel followed by electrophoresis and wet transfer of proteins to nitrocellulose membrane (30 V/overnight). Membranes were blocked with 5%

non-fat milk in TBS (Tris-buffered saline, BioShop, Burlington, Ontario, Canada) + 0.1% (v/v) Tween-20 (BioShop, Burlington, Ontario, Canada) for 1.5 h at room temperature. Primary antibodies diluted in blocking solution: rabbit anti-HO-1 (ADI-SPA-894, Enzo Life Science, Farmingdale, NY, USA, 1:500), mouse anti- α -tubulin (CP06, Calbiochem, San Diego, CA, USA, 1:1000) were used for overnight incubation at 4°C. Next day, membranes were washed and incubated with secondary antibodies conjugated with HRP: goat anti-mouse (BD Pharmingen, San Diego, CA, USA, 1:10000) and goat anti-rabbit (Cell Signaling Technology, Danvers, MA, USA, 1:5000) for 1 h at room temperature. After series of washings, luminescent substrate for HRP activity (Immobilon Chemiluminescent HRP Substrate, Merck Millipore, Billerica, MA, USA) was added for 10 minutes and membranes were manually developed on X-ray film.

Immunofluorescence. Cells were fixed in 4% paraformaldehyde for 10 minutes, permeabilized with 0.05% (v/v) TritonX-100 for 5 minutes and incubated for 30 minutes in 0.25% (w/v) glycine in PBS. Afterwards, cells were blocked in 10% (v/v) goat serum (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 1 h at room temperature followed by incubation (overnight at 4°C) with primary antibody rabbit anti-HO-1 (ADI-SPA-894, Enzo Life Science, Farmingdale, NY, USA) diluted 1:200 in 1% (v/v) goat serum in PBS. Next day, cells were incubated with secondary antibody goat anti-rabbit Alexa Fluor 488 or 568 (Thermo Fisher Scientific, Waltham MA, USA) diluted 1:500 in 1% (v/v) goat serum together with 10 $\mu\text{g}/\text{mL}$ Hoechst (Sigma-Aldrich, St. Louis, MO, USA) for 2 h at room temperature, visualized

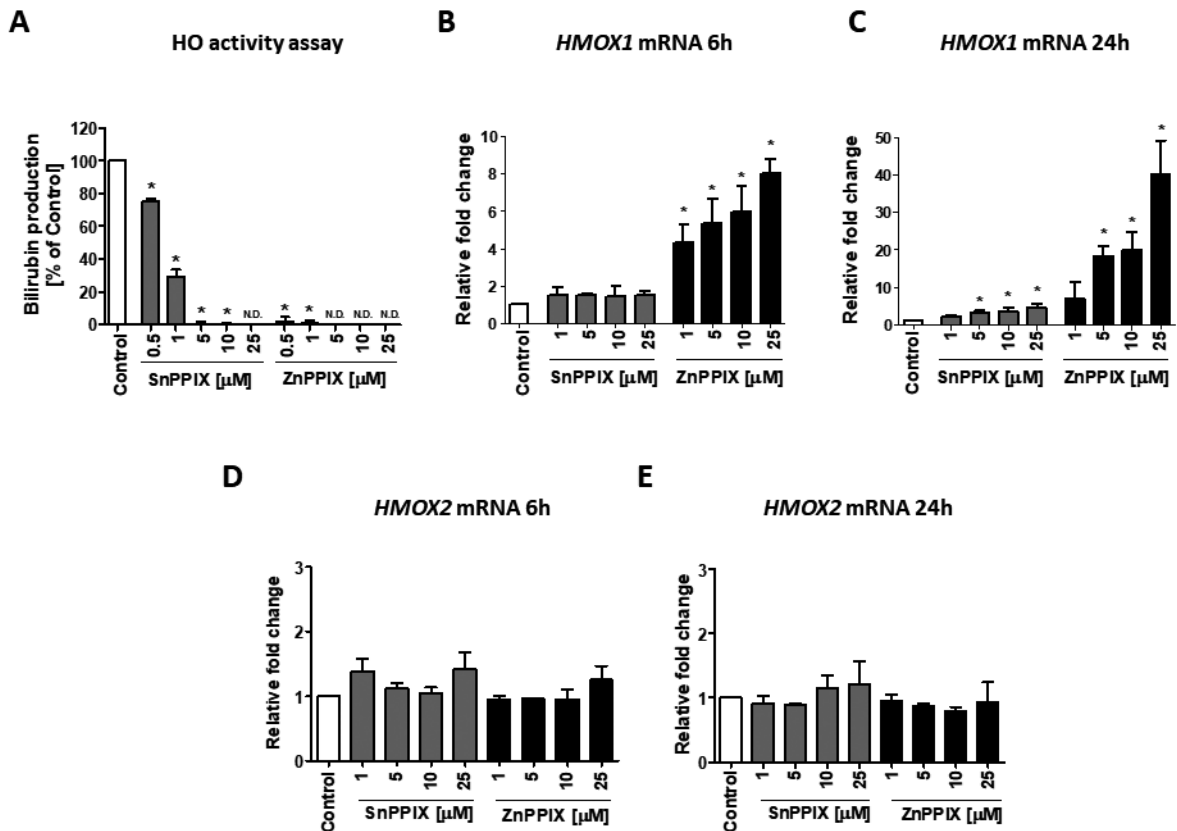


Figure 1. Inhibition of HO activity using SnPPIX and ZnPPIX affects *HMOX1* but not *HMOX2* mRNA level in 293T cell line. HO activity after 6 h stimulation with SnPPIX/ZnPPIX shown as the percentage of control (mean \pm SD) (A). *HMOX1* and *HMOX2* mRNA after 6 h (B, D, respectively) and after 24 h (C, E, respectively) stimulation with 1–25 μM SnPPIX/ZnPPIX calculated as relative fold change vs. control (mean \pm S.D.).

under fluorescent microscope and analyzed using *ImageJ* program.

Statistical analysis. All experiments were performed in duplicates or triplicates and were repeated two or three times. Results are presented as a mean \pm S.D. (standard deviation). Statistical analysis was performed in GraphPad Prism Software using Student's *t*-test or one-way ANOVA. Results were considered statistically significant at $p < 0.05$

RESULTS

Protoporphyrins inhibit HO activity but upregulate HO-1 mRNA and protein

Protoporphyrins, SnPPIX and ZnPPIX are widely used inhibitors of HO activity. In our hands, 6 h stimulation with either inhibitor led to potent decrease in bilirubin production by 293T cells, with much stronger effect of ZnPPIX (Fig. 1A). Nonetheless, already 6 h stimulation with ZnPPIX resulted in significant induction of *HMOX1* mRNA (Fig. 1B). After longer time (24 h), the stimulatory effect of SnPPIX on *HMOX1* expression was also visible (Fig. 1C), but was not as pronounced as after ZnPPIX treatment which was even further increased in comparison to 6 h stimulation (Fig. 1C). Noteworthy, 6 h (Fig. 1D) and 24 h (Fig. 1E) treatment with protoporphyrins had no significant effect on the mRNA expression of constitutive form of HO, *HMOX2*. The induction of HO-1 protein by protoporphyrins was confirmed by Western blot (Fig. 2A) and immunofluorescent staining (Fig. 2B).

shHO-1 fail to abolish HO activity induction upon hemin treatment

293T cells were transduced with lentiviral vectors encoding shRNA against HO-1 (shHO-1 A-D) and scrambled shRNA (further referred to as control cells) and

the efficiency of the transduction was monitored using fluorescent microscope and flow cytometer (Fig. 3A). After sorting GFP⁺ cells, HO-1 expression was evaluated. We observed a decrease in basal *HMOX1* mRNA in cells transduced with three out of four tested shRNAs (sequences B-D, Fig. 3B); however, 6 h stimulation with hemin, HO-1 inducer, caused potent induction of *HMOX1* in cells transduced either with scrambled shRNA or all shHO-1 (Fig. 3C). *HMOX2* mRNA was affected neither by shHO-1 transduction (Fig. 3D) nor by hemin treatment (Fig. 3E). Moreover, basal HO-1 protein level was decreased in cells transduced with shHO-1 B-D sequences (Fig. 4A, B). Hemin-induced HO-1 upregulation was observed in shHO-1 KD cells but it was not as potent as in control cells, as shown by immunofluorescent staining (Fig. 4A) and Western blot (Fig. 4B). Finally, HO activity was not significantly changed in basal conditions (Fig. 4C). In accordance with the mRNA and protein results after hemin stimulation, all shHO-1 cells showed increase in HO activity similar to cells transduced with scrambled shRNA (Fig. 4D).

CRISPR/Cas9-mediated gene editing of *HMOX1* locus results in HO-1 deficiency

In order to obtain HO-1 knock-out cells, 293T cells were transiently transfected with vectors coding for two distinct sgRNAs that target second exon of human *HMOX1* locus (Fig. 5A). We chose second exon because it encodes histidine 25, which is prerequisite for HO-1 catalytic activity and plasmids may be used in future to create mutant clones with catalytically inactive HO-1 instead of *HMOX1* knock-out clones. Upon transfection, the cells were selected with puromycin for 24 h and seeded on 96-well plate to obtain single cell-derived clones. After further amplification of cells, DNA was isolated and CELI mismatch detection assay was performed to evaluate the presence of mutations in *HMOX1* amongst the obtained clones (Fig. 5B). Of

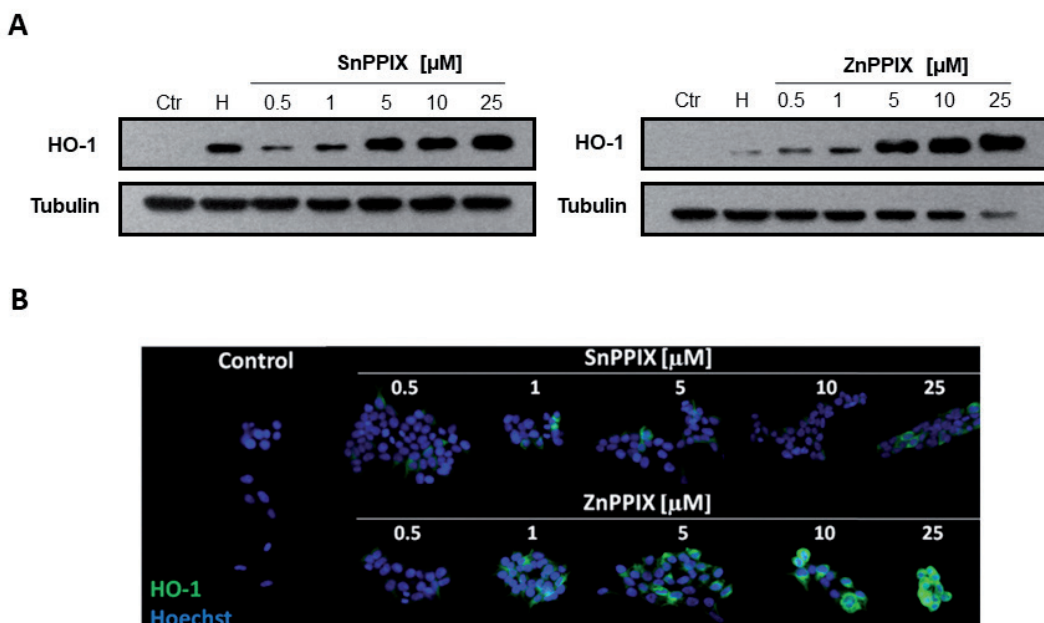


Figure 2. Pharmacological inhibitors of HO activity potently increase HO-1 protein level.

Western blot analysis of HO-1 protein after 24 h stimulation with inhibitors (0.05–25 μ M) and 10 μ M hemin (H) (A). Immunofluorescent detection of HO-1 protein (green) after 24 h stimulation with protoporphyrins (0.05–25 μ M), counterstained with Hoechst (B). * $p < 0.05$ vs. control.

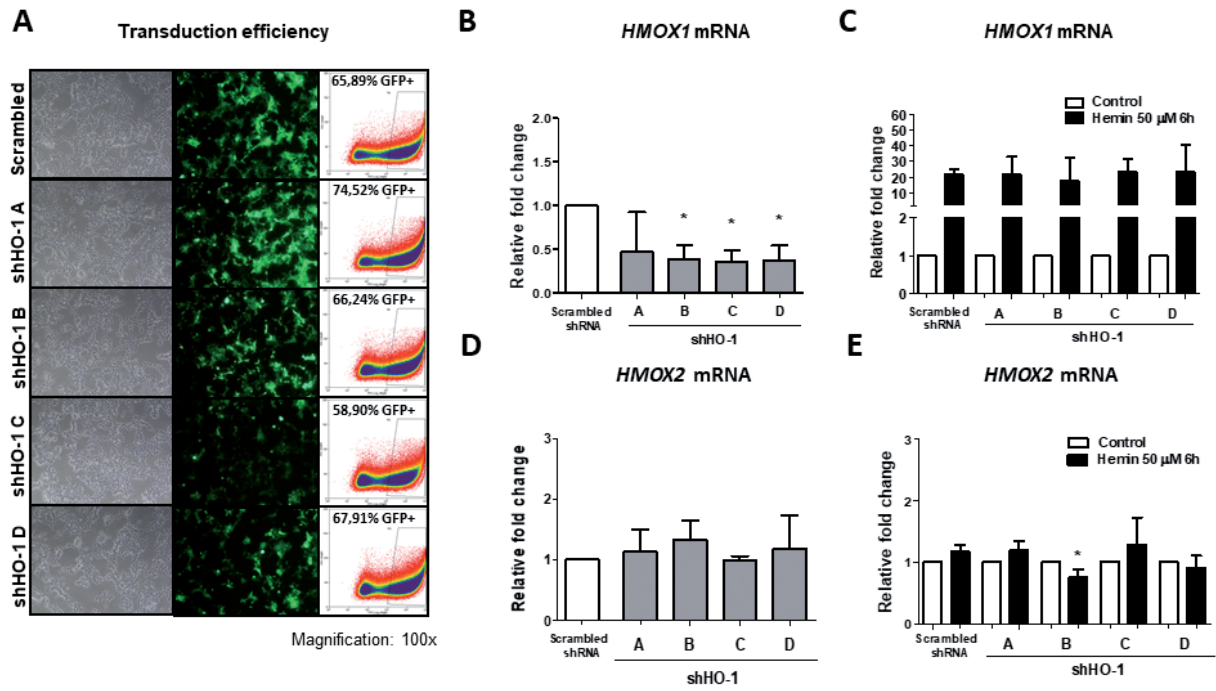


Figure 3. shRNA-mediated *HMOX1* silencing fails to abolish *HMOX1* induction after hemin treatment. Representative pictures of cells transfected with lentiviral vectors encoding shRNA against *HMOX1* (100x) and percentage of GFP+ cells (A). *HMOX1* and *HMOX2* (B, D, respectively) expression after transduction with shHO-1 A-D and scrambled shRNA, presented as relative fold change vs. control (mean \pm S.D.).

note, among 31 tested clones, 18 harbored mutations in *HMOX1* locus (Fig. 5C).

Due to CRISPR/Cas9-mediated introduction of premature stop codons and subsequent degradation of defective transcripts in nonsense-mediated decay process,

we observed diminished level of *HMOX1* transcript in several clones harboring mutations in *HMOX1* locus. The most pronounced difference was detected in clones 4.4, 4.8 and to a lesser extent in clones 4.14 and 4.20 (Fig. 6A). Hemin-induced *HMOX1* expression was

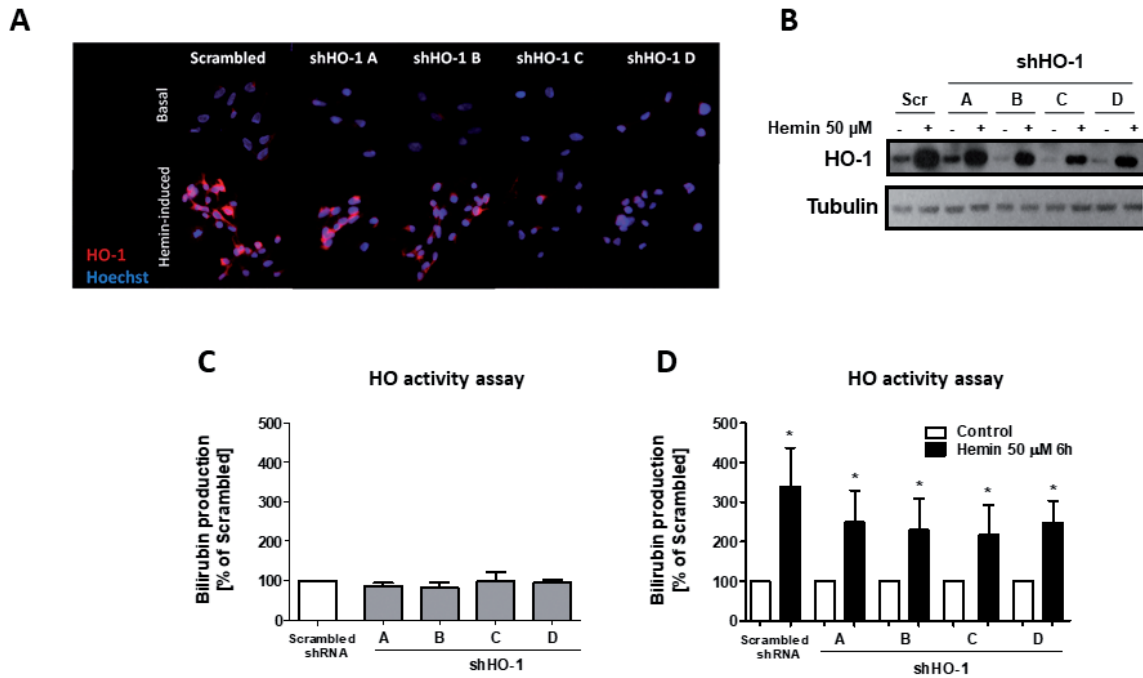


Figure 4. shRNA-mediated silencing of *HMOX1* partially decreases basal and hemin-induced protein level, with no effect on HO-1 activity level. HO-1 protein level assessed in basal and hemin-induced (6 h stimulation) conditions by immunofluorescent staining (red) counterstained with Hoechst (A) and by Western blot (B). HO activity under basal (C) and hemin-stimulated (6 h) conditions (D). HO activity under basal conditions is shown as percentage of control and at hemin-induced conditions as percentage of basal conditions for appropriate cell line (mean \pm S.D.). * $p < 0.05$ vs. control.

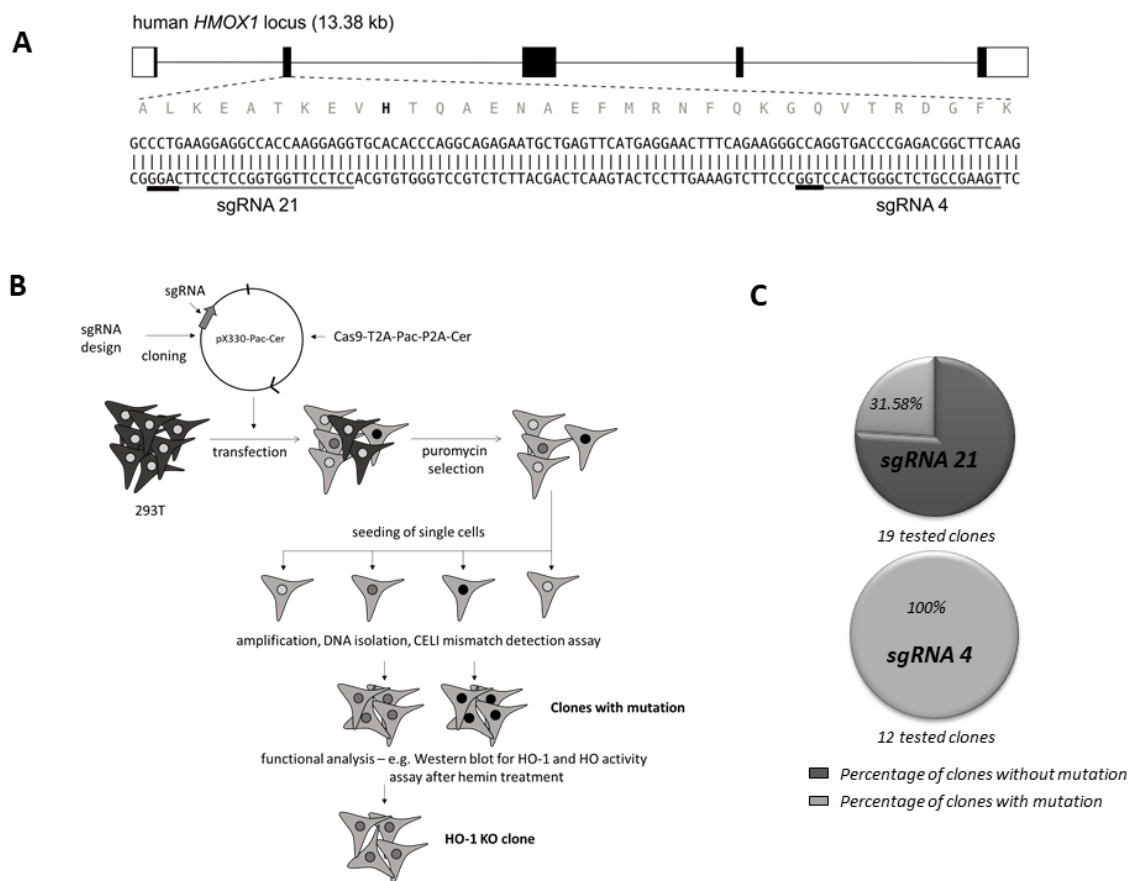


Figure 5. Efficient CRISPR/Cas9-mediated introduction of mutations in *HMOX1* in 293T cell line.

Schematic representation of human *HMOX1* locus showing the location of *HMOX1*-targeting sgRNAs (grey lines; PAM sequences are shown in black). Black lines represent introns and rectangles represent exons. DNA sequence of fragment of exon 2 is shown below; sequence of the protein translated from this fragment is given immediately above the nucleotide sequence; H25 is shown in bold (A). Designed sgRNAs were further utilized to generate HO-1 knockout clones of 293T cells according to general scheme of CRISPR/Cas9-mediated gene editing (B). Efficient introduction of mutations in *HMOX1* in derived clones was confirmed using CELL mismatch detection assay (C). The results are presented as percentage of clones with detected mutations upon utilization of two sgRNAs sequences.

blunted in several clones, in comparison to hemin-stimulated control cells (Fig. 6B). Basal *HMOX2* mRNA level was not affected in majority of clones (Fig. 6C) whereas some inhibition was detected in hemin-stimulated cells (Fig. 6D). Both basal and hemin-induced HO-1 protein level was potentially decreased mostly in clones 4.4, 4.8, 4.10 and 4.14 (Fig. 7A, 7B). Most probably, those clones harbor frameshift mutation in all *HMOX1* loci. HO activity, which is a combined activity of HO-1 and HO-2, was not changed in basal conditions (Fig. 7C) (except for the clone 4.8), but most importantly, lack of response to hemin stimulation (as the result of blocked HO-1 activity) in all clones was observed (Fig. 7D).

CRISPR/Cas9-mediated HO-1 depletion affects 293T cells viability and growth

HO-1 depletion in 293T cells caused slight, but significant drop in the cell growth in clones 4.8, 4.10, 4.14, as checked by CyQUANT analysis (Fig. 8A). Furthermore, tested clones (4.8 and 4.10) exhibited decreased viability in basal conditions and significantly higher susceptibility to 0.5 mM H_2O_2 stimulation in comparison to control cells (Fig. 8B). Additionally, clonogenic potential of 293T cell line was significantly impaired by HO-1 depletion in comparison to control cells (Fig. 8C).

DISCUSSION

Due to the growing body of evidence suggesting the relevance of HO-1 inhibition as a possible therapeutic strategy, especially in tumour biology, we decided to provide a comprehensive comparison of pharmacological and genetic approaches of HO-1 silencing in a model cell line, human embryonic kidney cells 293T.

Experimental and medical application of metalloporphyrins is limited mostly due to their poor specificity, low bioavailability, and the modulation of pathways not directly related to HO (Grundemar & Ny, 1997; Schulz *et al.*, 2012). Accordingly, ZnPPiX has been demonstrated to regulate the indoleamine-2,3-dioxygenases activity (Metz *et al.*, 2010), cyclin D1 expression (Rahman *et al.*, 2009), and Wnt/ β -catenin pathway (Wang *et al.*, 2013). We have previously shown that SnPPiX and ZnPPiX may oppositely regulate the activity of inducible nitric oxide synthase (iNOS), as SnPPiX augmented whereas ZnPPiX decreased production of NO in vascular smooth muscle cells as well as in macrophages and those effects were HO-1 independent (Jozkowicz & Dulak 2003).

As reported in the present study, both SnPPiX and ZnPPiX almost completely abolished total HO activity, emphasizing their unspecificity towards HO isoforms. At the same time, we observed strong upregulation of HO-1 both at mRNA and protein levels. This effect was

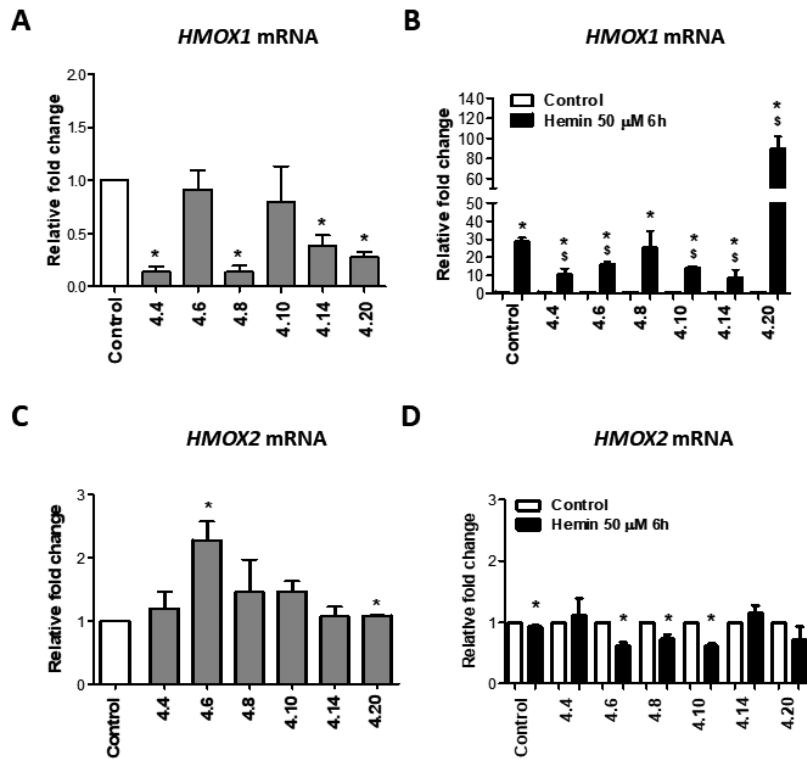


Figure 6. Genetic inhibition of *HMOX1* in 293T using CRISPR/Cas9 system significantly decreases *HMOX1* but not *HMOX2* mRNA level both under basal and hemin-induced conditions. *HMOX1* and *HMOX2* expression in control clone and in clones after CRISPR/Cas9-mediated HO-1 excision in basal (A, C, respectively) and in hemin-induced conditions (B, D, respectively). Basal level was calculated as relative fold change vs. control whereas hemin-induced *HMOX1* expression is shown as relative fold change vs. basal conditions for each clone (mean \pm S.D.).

also reported in other cell types and tissues (Loboda *et al.*, 2015). It has been proposed that such a dual action might be related to the fact that metalloporphyrins, acting as the analogues of heme, could remove transcrip-

tional Bach1 repressor, induce *HMOX1* transcription through Nrf2 activation, and simultaneously inhibit its activity by binding to the catalytic pocket of HO-1 (Ogawa *et al.*, 2001). Additionally, it was reported that

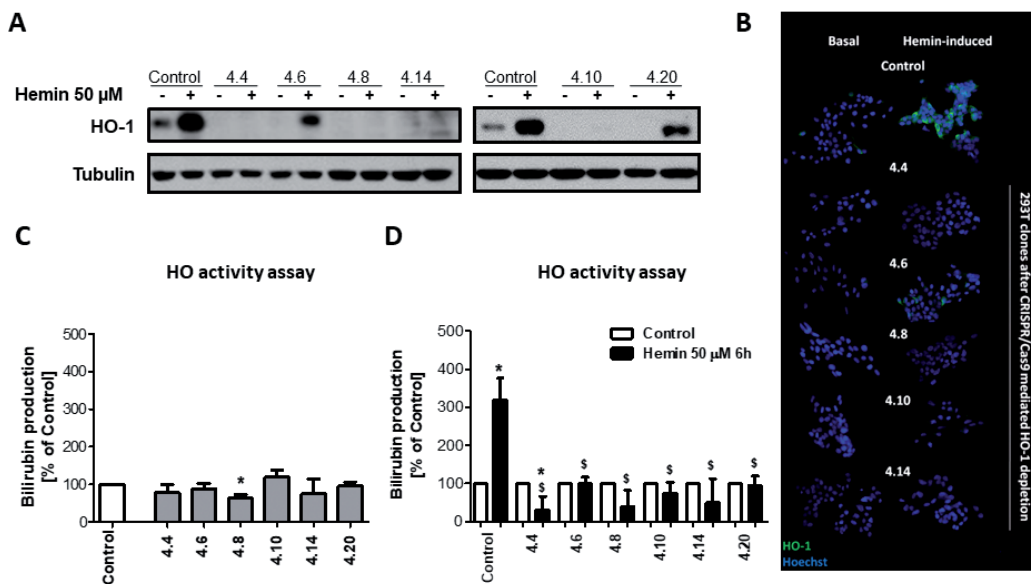


Figure 7. Genetic inhibition of *HMOX1* in 293T using CRISPR/Cas9 system leads to potent decrease in HO-1 protein level both under basal and hemin-induced conditions as well as on activity level after treatment with hemin. Inhibition of HO-1 protein level by CRISPR/Cas9-mediated HO-1 excision checked by immunofluorescent staining (A) and Western Blot (B) both under basal conditions and after 6 h stimulation with 50 μ M hemin. HO activity in basal conditions (C) and after 6 h stimulation with 50 μ M hemin (D). HO activity at basal conditions shown as percentage of control and hemin treatment as percentage of basal conditions for appropriate clone (mean \pm S.D.). * p <0.05 vs. control; \S p <0.05 vs. control cells treated with hemin.

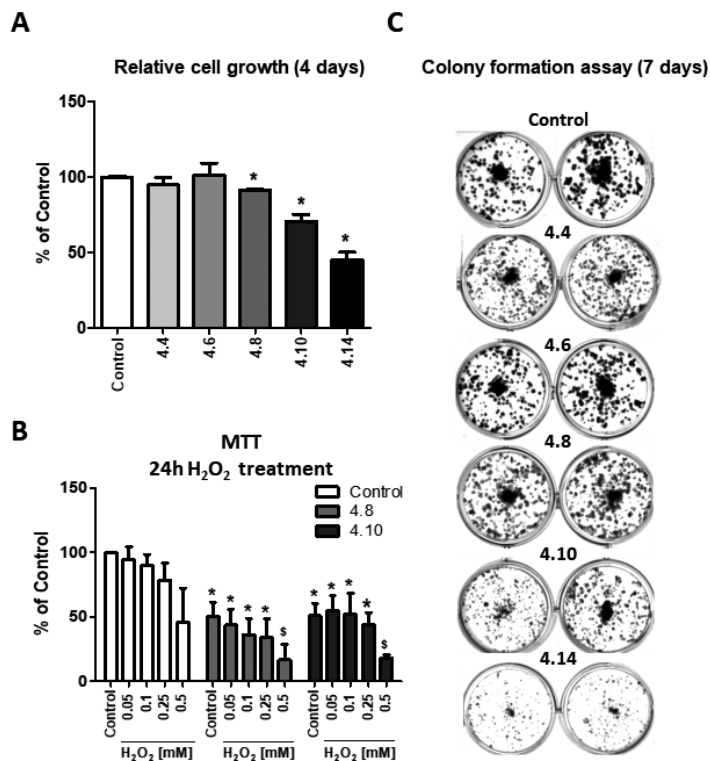


Figure 8. The effect of *HMOX1* inhibition by CRISPR/Cas9 system on viability, proliferation and clonogenic potential of 293T cell line. CyQUANT assay to estimate relative growth rate was performed after 4 days from seeding. The results are presented as percentage of control (mean \pm S.D.). **(A)** MTT analysis on cells stimulated with H₂O₂ (0.05–0.5 mM) for 24h and calculated as percentage of control (mean \pm S.D.). **(B)** Clonogenic potential checked 7 days after seeding – representative pictures **(C)**. * $p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. control cells of each clone.

ZnPPiX mediates binding of early growth response-1 (Egr-1) to the promoter of HO-1, which then induces its expression (Yang *et al.*, 2001). Summarizing, due to HO-independent, unspecific effects, results obtained using metalloporphyrins should be analysed with caution and the involvement of the HO-1 pathway should be always confirmed by more specific methods.

Despite high transduction efficiency in 293T cells obtained in the shRNA approach, most of the tested shRNA sequences decreased *HMOX1* mRNA level only to about 35% of that in control. This partial effect can be cell type-specific but also connected to some internal protective mechanism in cells. The increase in MOI of used vectors would be one option to augment the effectiveness; however, it might also lead to toxicity and off-target effects. Moreover, it appeared important to test few different shRNA sequences, since the efficacy of each of them can vary (Alaoui-Jamali *et al.*, 2009). It was also clearly visible in our hands that shHO-1 sequence A only slightly diminished *HMOX1* expression with no changes on protein level. Fortunately, none of tested sequences influenced *HMOX2* transcript, which suggests specificity regarding HO isoforms. This also explains the lack of the effect on basal HO activity, which probably reflects mostly the activity of HO-2, the isoform predominant in physiological conditions, rather than HO-1. Furthermore, significant induction of *HMOX1* mRNA, protein, and activity in response to hemin stimulation was still visible and can be related to insufficient inhibition of newly transcribed *HMOX1* mRNA by shRNA. The lack of possibility to inhibit the increase in HO activity in response to the stimuli is an important and major drawback of this ap-

proach as HO-1 is easily-inducible enzyme (Ferrandiz & Devesa 2008).

In the present study we have clearly shown the advantage of CRISPR/Cas9 technology over shRNA method. Although we observed differences in efficiency of HO-1 depletion between tested clones which can be related to natural variability between cells or some pitfalls of CRISPR/Cas9 technology (Peng *et al.*, 2016) and emphasize the need for careful selection of clones used for further studies, our data clearly demonstrate the usefulness of this method. It has to be stressed that despite the fact that *HMOX1* transcript which is most probably mutated, can be still present in cell clones transfected with CRISPR/Cas9 targeting *HMOX1*, the functionality of the protein is lost. HO-1 protein level in CRISPR/Cas9-derived cell clones was diminished, both in basal and hemin-induced conditions and, what is most important, no increase in HO activity upon stimulation with potent inducer of HO-1, hemin, was observed. This reflects the necessity of validation of the obtained results not only at mRNA or protein levels, but essentially based on the biological functionality, which in our case is a real indicator of the effectiveness of the CRISPR/Cas9 approach.

Importantly, the lack of HO-1 in 293T cells exerts serious biological consequences, including decreased clonogenic potential and cell proliferation as well as diminished cell viability. Moreover, cells with HO-1 deficiency are more susceptible to H₂O₂ stimulation, which is connected to the cytoprotective role of HO-1 against stressful conditions. Therefore, HO-1 may be a good anticancer target. Indeed, a growing body of evidence suggests the emergent role of HO-1 in various types of cancer, including melanoma, renal cell carcinoma, pancreatic can-

cer, and many others. Elevated levels of HO-1 in cancer cells in comparison to cells from healthy tissues has been associated with different aspects of tumour biology, leading to progression of the disease (Loboda *et al.*, 2015). Furthermore, expression of HO-1 can be induced upon anticancer treatment, which often results in decreased effectiveness. Nonetheless, the exact role of HO-1 in tumour biology is multifaceted, since the opposite, anticancer activity of HO-1 was described, for example, in the case of breast or prostate cancer. Thus, although HO-1 might be considered as a therapeutic target, it has to be taken into account that inhibition of HO-1 not in every cancer cell type will be desired. Even more complicated is the fact that contradictory results were obtained within the same type of cancer, which might be at least partially related to different experimental settings. Especially in the case of metalloporphyrins, caution has to be taken in the interpretation of obtained results, since they might not be directly related to the activity of HO-1. Moreover, when considering the shRNA approach, off-target effects may result in the misinterpretation of observed results (Loboda *et al.*, 2015). Thus, we do believe that CRISPR/Cas9 technology may represent the most reliable tool to study specific gene function. Moreover, to our best knowledge, depletion of HO-1 by CRISPR/Cas9 technology has not been studied so far, and here we report that it can provide an alternative method to study biological effect of HO-1 inhibition in many areas of research, including tumour biology, which is now extensively studied by our group.

Conflict of interest statement

The authors declare that there is no conflict of interests.

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