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Hemin Reduces Cellular Sensitivity to Imatinib and Anthracyclins Via Nrf2

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Abstract Heme plays an important biomodulating role in various cell functions. In this study, we examined the effects of hemin on cellular sensitivity to imatinib and other anti-leukemia reagents. Hemin treatment of human BCR/ABL-positive KCL22 leukemia cells increased IC_{50} values of imatinib, that is, the drug resistance, in a dose-dependent manner without any change in the BCR/ABL kinase activity. Imatinib-induced apoptosis was also suppressed by hemin treatment in KCL22 cells. Hemin treatment increased the activity of γ-glutamylcysteine synthetase (γ-GCS) light subunit gene promoter, which contains a Maf recognition element (MARE). Protein levels of γ-GCS and heme oxygenase-1 (HO-1), two MARE-containing genes, were also increased after hemin treatment. Knockdown of Nrf2 expression by RNA interference largely abolished the effect of hemin on imatinib-treated cells, suggesting that Nrf2 recognition of MARE is essential for the hemin-mediated protective effect. Similar to hemin, treatment of cells with δ-aminolevulinic acid (δ-ALA), the obligatory heme precursor, also increased IC_{50} values of imatinib. In contrast, inhibition of cellular heme synthesis by succinylacetone increased the sensitivity of cells to imatinib in two imatinib-resistant cell lines, KCL22/SR and KU812/SR. Hemin treatment also decreased the sensitivity of cells to four anthracyclins, daunorubicin, idarubicin, doxorubicin, and mitoxantrone, in BCR/ABL-negative leukemia U937 and THP-1 cells, as well as in KCL22 cells. These findings thus indicate that cellular heme level plays an important role in determining the sensitivity of cells to imatinib and certain other anti-leukemia drugs and that the effect of heme may be mediated via its ability to upregulate Nrf2 activity. J. Cell. Biochem. 104: 680–691, 2008.

Key words: heme; imatinib; anthracyclin; Nrf2; KCL22; drug resistance

Imatinib mesylate (Imatinib, Novartis Pharma (Basel, Switzerland)), an ABL tyrosine kinase inhibitor, induces a substantial clinical response in patients with BCR/ABL-positive leukemia [Deininger et al., 2005]. A recent study showed that 87% of patients with chronic myeloid leukemia (CML) who were treated with imatinib in the chronic phase achieved complete cytogenetic response (CCR) at 36 months [Roy et al., 2006]. However, its efficacy in an aggressive state of the disease such as in blast crisis, or in acute lymphoblastic leukemia is not satisfactory due to development of resistance to the drug [Druker et al., 2001; Sawyers et al., 2002]. The mechanisms by which drug resistance develops may be very complex but appear to occur both in BCR/ABL kinase activity-dependent and -independent manners.

Heme is known to exert important biomodulating effects on various cellular functions [Sassa and Nagai, 1996]. For example, aside from being the prosthetic group for hemoproteins, heme is involved in the regulation of

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protein synthesis, cGMP formation, heme biosynthesis as well as heme metabolism. Heme also directly regulates the activity of certain transcription factors, for example, Nrf2 and Bach1, members of the CNC family of basic region-leucine zipper transcription factors [Moi et al., 1994; Oyake et al., 1996], which contain a common gene regulatory element, Maf recognition element (MARE) [Shibahara, 2003; Dhakshinamoorthy et al., 2005]. MARE is known to be present in the promoter region of several detoxifying and anti-oxidative stress genes such as γ-glutamylcysteine synthetase (γ-GCS), which is the rate-limiting enzyme of the glutathione (GSH) synthetic pathway, and in HO-1, the 32 kDa heat-shock protein as well as the rate-limiting enzyme in heme catabolism [Venugopal and Jaiswal, 1996, 1998; Itoh et al., 1997; Ishii et al., 2000; Shibahara, 2003]. Nrf2 is known to bind to MARE, including the γ-GCS light subunit promoter region [Tarumoto et al., 2004] and HO-1 [Shibahara, 2003]. With an increase in cellular heme concentration, Bach1 DNA binding activity is rapidly decreased, leading to increased Nrf2-mediated gene expression via decreased Bach1 transrepression activity [Shibahara, 2003].

We previously demonstrated that the sensitivity of imatinib-resistant cells to imatinib can be increased by ascorbic acid treatment, which suppresses Nrf2 expression and thus decreases in the levels of γ-GCS light subunit mRNA and GSH [Tarumoto et al., 2004]. GSH are also known to be involved in resistance to some anticancer drugs [Iida et al., 1999; Estrela et al., 2006]. Other studies showed that hemin and erythropoietin, which induces hemoglobin formation in erythroid progenitor cells, suppressed imatinib-induced cell death in K562 leukemia cells [Kirschner and Baltensperger, 2003; Bonovolias et al., 2006]. Hemin has also been shown to prevent cytotoxicity or apoptosis induced by doxorubicin in leukemia cell lines, and is thought that its effect was presumably exerted by its inhibition of mitochondrial cytochrome c oxidase [Tsiftsoglou et al., 1986; Papadopoulou and Tsiftsoglou, 1996]. Furthermore, increased expression of HO-1, which regulates cellular heme and iron concentrations, is also known to prevent cell death [Ferris et al., 1999]. Thus, it seems important to investigate whether heme influences cellular sensitivity to imatinib and conventional anti-leukemic agents and, if so, how it does it.

In this study, we investigated the effect of hemin treatment on the sensitivity of human leukemia cells to imatinib and other conventional anti-leukemic agents. Our results show that heme is critically involved in determining cellular sensitivity to imatinib and that its effect is at least in part mediated via upregulation of Nrf2 function. Similar to imatinib, sensitivity to certain other conventional anti-leukemia drugs, particularly anthracyclins, is also suppressed by hemin treatment. These results thus suggest that heme plays an important role in determining the sensitivity of leukemia cells to imatinib and to certain other anti-leukemia agents.

MATERIALS AND METHODS

Cell Lines

KCL22 is a BCR/ABL-positive cell line established from peripheral blood of a patient with CML in blast crisis [Kubonishi and Miyoshi, 1983]. KCL22/SR and KU812/SR are imatinib-resistant cell lines cloned from KCL22 and KU812, respectively [Ohmine et al., 2003; Miyoshi et al., 2005]. U937 and THP-1 are BCR/ABL-negative human myeloid leukemia cell lines [Sundstrom and Nilsson, 1976; Collins et al., 1977]. These cells were grown in RPMI1640 medium supplemented with 10% fetal bovine serum, penicillin G and streptomycin sulfate and split every 4 days. For determination of IC_{50}, cells were incubated in the presence of various concentrations of reagents for 96 h, followed by staining with trypan blue. Based on the number of trypan blue positive cells, a dose–response curve of cellular viability was prepared as a function of reagent concentration, and a concentration that gives rise to 50% cellular viability was designated as IC_{50}.

Reagents

Imatinib was purchased from Novartis Pharma. Hemin, δ-aminolevulinic acid (δ-ALA), buthionine sulfoximine (BSO), and succinylacetone were purchased from Sigma Chemical Co. (St. Louis, MO).

Flow Cytometry

Annexin-V-positive cells were counted using a MEBCYTO-Apoptosis Kit (MBL, Nagoya, Japan). Briefly, cells were collected and incubated with annexin-V-FITC and propidium iodide for 15 min and then analyzed by flow

cytometry using a FACScan Analyzer (Becton Dickinson, San Jose, CA). For cell cycle analysis, cells were incubated with propidium iodide for 30 min and analyzed by flow cytometry using a FACScan/CellQuest System (Becton Dickinson). The percentages of G_0/G_1, $S+G_2/M$ and sub-G_1 fractions were calculated using the ModFitLT 2.0 program (Verity Software, Topsham, ME).

Western Blot Analysis

Whole cell lysates were prepared from 1×10^7 cells according to the method described previously [Nagai et al., 1997]. Fifty micrograms of lysates was loaded onto 10% polyacrylamide gel and separated electrophoretically. Immunoblotting and detection by enhanced chemiluminescence were performed as described previously [Nagai et al., 1997]. Mouse monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase, which was used as an internal control, was purchased from Chemicon International (Temecula, CA). Rabbit polyclonal anti-Nrf2 (V-20), anti-BCR, and anti-γ-GCS light subunit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-caspase-3, anti-cleaved caspase-3, anti-caspase-7, anti-cleaved caspase-7, anti-caspase-9, anti-cleaved caspase-9, anti-PARP, anti-cleaved PARP, anti-Bcl-xL, anti-phospho BCR, anti-p44/42 (ERK1/2) MAP kinase, and anti-phospho p44/42 (ERK1/2) MAP kinase rabbit polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-HO-1 mouse polyclonal antibody was purchased from BD Biosciences.

Transfection and Luciferase Assays

Two micrograms of luciferase reporter plasmid fused to the human γ-GCS light subunit gene promoter region (pGCS-pro; containing ARE and AP-1 binding sites) and 1 μg of pRL-CMV (internal control) were co-transfected into KCL22 cells using TransFast™ Reagent (Promega Corp., Madison, WI), as described previously [Tarumoto et al., 2004]. Briefly, a total of 1×10^6 cells were incubated with the plasmids and TransFast™ in 1 ml of medium without serum for 1 h. Then 5 ml of fresh medium containing 10% serum was added and the cells were incubated in the presence or absence of 100 μM hemin for 24 h. Luciferase activity was determined using a Dual-Luciferase Reporter Assay System (Promega Corp.).

Transfection of Small Interfering RNA (siRNA)

One micrograms of Nrf2, GFP or control random siRNAs was transfected using Xtreme-GENE siRNA transfection reagent (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's protocol. Nrf2, GFP, and control random siRNAs were purchased from Qiagen, Inc. (Valencia, CA).

RESULTS

Hemin Treatment Decreases Sensitivity of KCL22 Cells to Imatinib

First, we examined the effect of hemin on the growth of KCL22 cells following treatment with imatinib. Hemin is ferric protoporphyrin IX, which is available as a chemical, and it is known to be converted to the heme moiety of hemoglobin in murine erythroleukemia cells [Granick and Sassa, 1978]. Hemin itself had no effect on the growth of KCL22 cells when added alone (data not shown). Addition of 1 μM imatinib virtually abolished cell growth, while addition of 100 μM hemin partially restored imatinib-mediated suppression of cell growth beyond 48 h (Fig. 1A). Consistent with these findings, hemin treatment increased IC_{50} values of KCL22 cells to imatinib in a dose-dependent manner (Table I). Similar to hemin, treatment of cells with δ-ALA, the obligate heme precursor, increased the IC_{50} value of imatinib (Table I). These findings suggest that the sensitivity of cells to imatinib is decreased by an increase in intracellular heme concentration.

Addition of imatinib resulted in an increase in G_0/G_1 cells (Fig. 1B), which is consistent with our previous finding that induction of G_0/G_1 arrest was the major reason for imatinib-induced growth inhibition of KCL22 cells [Komatsu et al., 2003]. However, imatinib treatment also slightly increased the percentage of sub-G_1 cells as well as the number of annexin-V-positive cells (Figs. 1B,C). These results suggest that, to certain extent, induction of apoptosis may also be involved in imatinib-induced growth inhibition, consistent with a view reported earlier [Brady, 2003].

While treatment of cells with hemin alone did not change percentages of G_0/G_1 and sub-G_1 cells ($P = 0.431$, $P = 0.236$, respectively, Fig. 1B), the imatinib-mediated increase in sub-G_1 cells or annexin-V-positive cells was significantly decreased by hemin treatment ($P = 0.024$, $P = 0.027$, respectively, Figs. 1B,C).

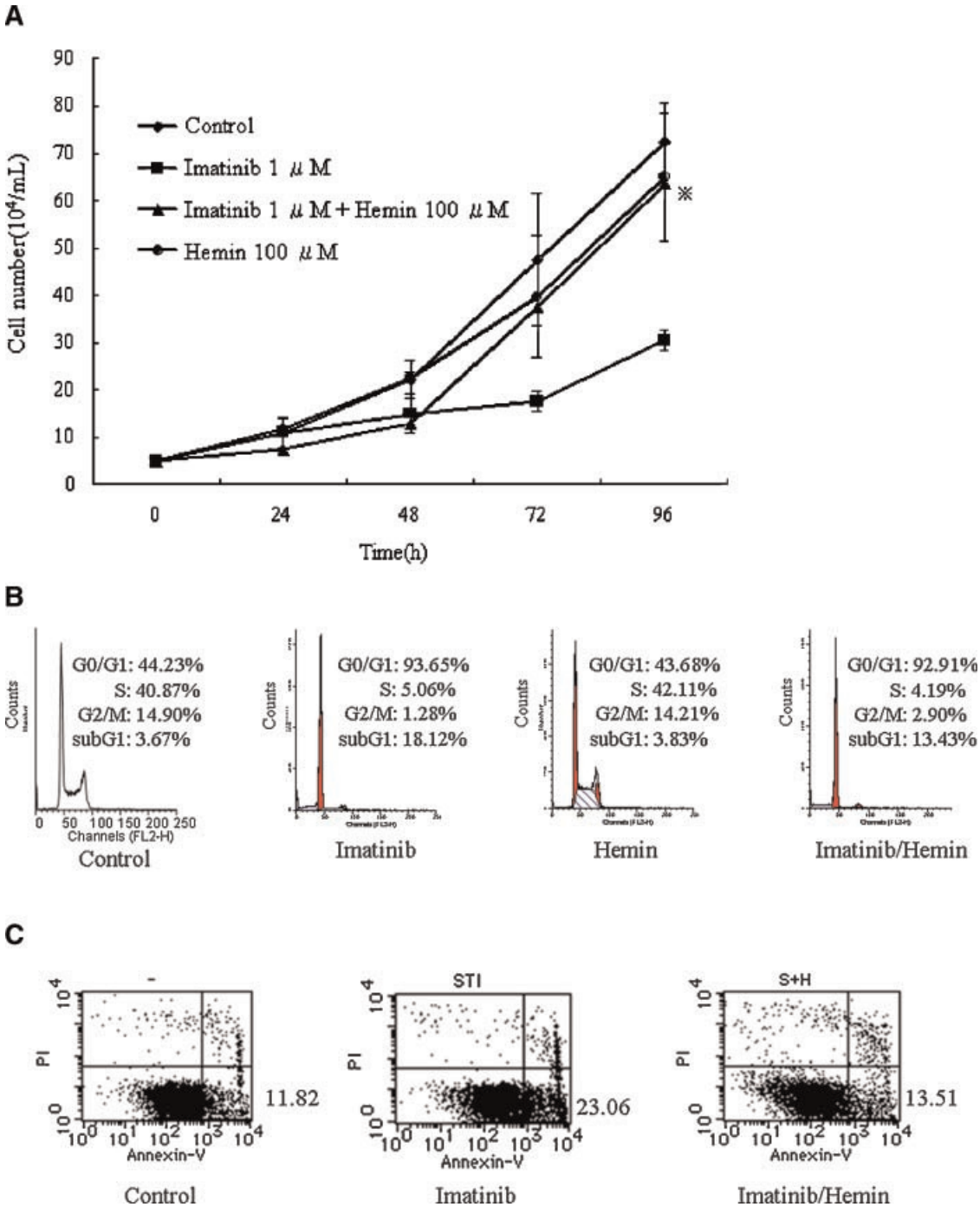

Fig. 1. Hemin treatment decreases the sensitivity of KCL22 cells to imatinib. **A**: Effect of hemin on imatinib-mediated growth inhibition. KCL22 cells were incubated with 1 μM imatinib or with a combination of 100 μM hemin and 1 μM imatinib. Cells were harvested at various time points as indicated in the figure. The number of viable cells was counted at each time point using trypan blue staining. Experiments were repeated three times. Error bars represent the standard deviation. Statistical analysis was carried out using Scheffe's test for comparison of the data between imatinib-treated cells and cells treated with a combination of imatinib and hemin (*$P < 0.05$). **B**: After 72 h of incubation of cells with 1 μM imatinib, or with imatinib and 100 μM hemin, cells were harvested and incubated with propidium iodide for 30 min and analyzed by flow cytometry with a FACScan/CellQuest system (Becton Dickinson). The results shown in the figure are representative of three independent experiments. **C**: Cells were treated with 1 μM imatinib or with imatinib and 100 μM hemin for 72 h. The number of annexin-V-positive cells was counted by flow cytometry as described in Section ''Materials and Methods.'' The results shown in the figure are representative of three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I. Effect of Hemin and ALA on IC_{50} Values of Imatinib in KCL22 Cells

	Concentration (μM)	IC_{50} value (μM)	Fold increase
Hemin	0	0.305 ± 0.087	1
	1	0.257 ± 0.076	0.84
	10	0.312 ± 0.088	1.02
	50	0.473 ± 0.218	1.55
	100	0.900 ± 0.137	2.95*
ALA	0	0.477 ± 0.005	1
	500	0.441 ± 0.002	0.92
	1,000	0.612 ± 0.017	1.28
	2,000	1.542 ± 0.187	3.23*

δ-ALA, δ-aminolevulinic acid.
Cells were incubated in the presence of various concentrations of hemin or ALA in triplicate for 72 h. Statistical analysis was carried out using Scheffe's test for comparison of the data between control cells and hemin or ALA-treated cells (*$P < 0.05$). Data are shown as means ± SEM of three independent experiments.

Consistent with these findings, hemin treatment suppressed imatinib-mediated induction of apoptosis-related molecules such as cleaved caspase-3, cleaved caspase-7, cleaved caspase-9, and cleaved PARP (Fig. 2A). The imatinib-mediated decrease in Bcl-xL levels was also restored when hemin was added. Furthermore, imatinib-mediated decreases in the level of cytochrome c in a mitochondrial fraction were restored by hemin (Fig. 2B), suggesting that imatinib-induced cytochrome c release from mitochondria was inhibited by hemin treatment. These results suggest that hemin treatment of KCL22 cells decreases cellular sensitivity to imatinib by inhibition of apoptosis signaling. The level of phosphorylated BCR/ABL was decreased by imatinib irrespective of the presence or absence of hemin (Fig. 2C), suggesting that hemin-induced decrease in imatinib-sensitivity does not involve BCR/ABL kinase activity.

Hemin Treatment of KCL22 Cells Increases Nrf2-Mediated Gene Expression

We previously showed that the Nrf2 transcription factor is involved in the imatinib resistance in imatinib-resistant KCL22/SR cells [Tarumoto et al., 2004]. Since Nrf2 function is known to be directly regulated by heme, it is possible that hemin-mediated decrease in cellular sensitivity to imatinib may be brought about by changes in Nrf2 activity. To determine whether hemin influences Nrf2-mediated gene expression, we examined the effect of hemin on

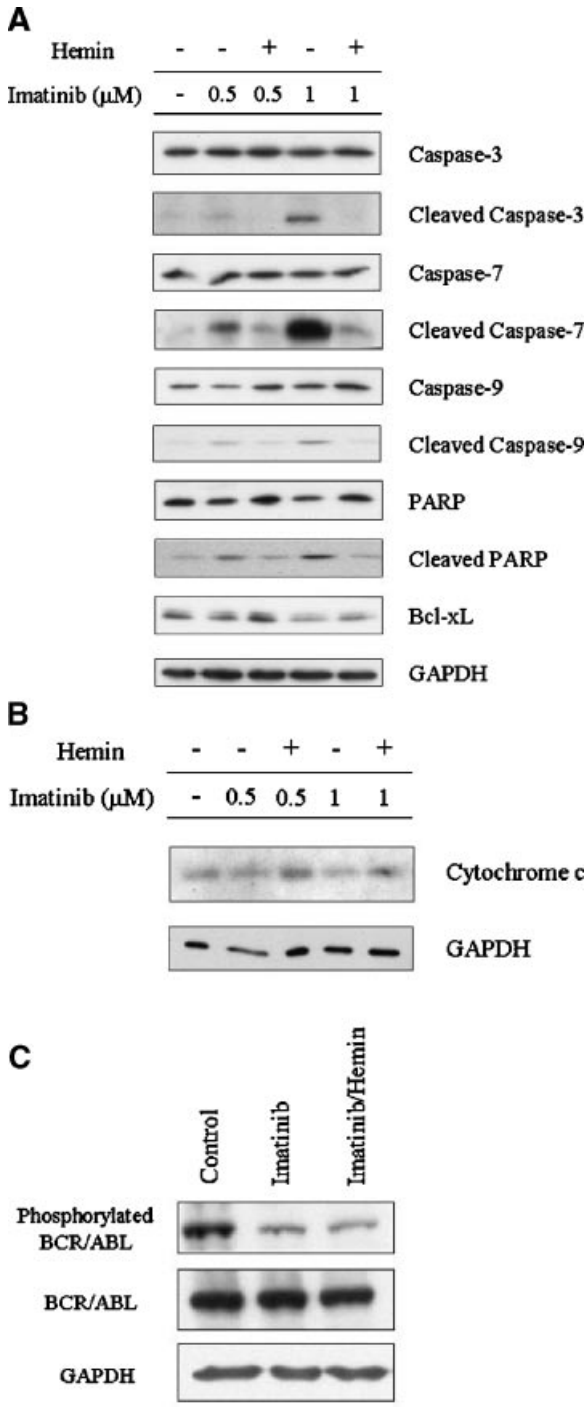

Fig. 2. Hemin treatment suppresses imatinib-induced apoptosis without influencing BCR/ABL function. **A**: Cells were treated with imatinib or with imatinib and hemin for 24 h at concentrations shown in the figure. Cell lysates were prepared and subjected to Western blot analysis. **B**: Cells were treated with imatinib or with imatinib and hemin for 12 h at concentrations shown in the figure. Mitochondrial lysates were prepared and subjected to Western blot analysis. **C**: Cells were treated with 1 μM imatinib or with a combination of imatinib and 100 μM hemin for 6 h. Immunoblot analyses using human anti-phospho BCR and anti-BCR antibodies were performed as described in Section ''Materials and Methods.''

MARE-mediated γ-GCS gene promoter activity by a luciferase reporter assay. When pGCS-pro, which contains a MARE site [Tarumoto et al., 2004], was transfected into KCL22 cells, the level of luciferase activity was increased by hemin treatment by approximately fivefold over the control level (Fig. 3A). In contrast, hemin had no effect on luciferase activity when cells were transfected with a promoterless pGL3-Basic. Similar results were obtained when KU812, another BCR/ABL-positive cell line, was used instead of KCL22 (data not shown).

To clarify the involvement of Nrf2 in the hemin-mediated upregulation of γ-GCS gene promoter activity, we examined the effect of silencing Nrf2 expression by siRNA. When KCL22 cells were transfected with Nrf2 siRNA, Nrf2 protein levels decreased to an extremely low level for 72 h (Fig. 3B). In cells transfected with Nrf2 siRNA, γ-GCS gene promoter activity was markedly suppressed, both in the presence and absence of hemin (Fig. 3A). It was significantly increased by hemin treatment in cells treated with control random siRNAs. These results indicate that hemin treatment of cells increased γ-GCS gene promoter activity by upregulating Nrf2 function.

Consistent with these results, hemin treatment of cells increased the protein level of γ-GCS (Fig. 3C). Furthermore, hemin treatment increased the protein level of HO-1, which is also under the control of Nrf2 through MARE [Dhakshinamoorthy et al., 2005]. It is of interest to note that HO-1 was reported to be a novel BCR/ABL-dependent survival factor of CML cells [Mayerhofer et al., 2004]. In contrast, imatinib treatment had no effect on the levels of γ-GCS and HO-1 proteins (Fig. 3C). Hemin-mediated induction of γ-GCS and HO-1 protein levels was suppressed by Nrf2 siRNA transfection, whereas transfection of cells with control GFP siRNAs had no effect (Fig. 3D). These results thus clearly indicate that the hemin-induced effect is due to upregulation of Nrf2, though it is not necessarily reflected in an increase in the total Nrf2 protein level.

Nrf2 Is Essential in the Hemin-Mediated Decrease in Imatinib Sensitivity

Next, we examined the effect of silencing Nrf2 expression by siRNA on imatinib-induced changes in apoptosis-related molecules. As shown in Figure 4, transfection of Nrf2 siRNA substantially suppressed the effect of hemin on the imatinib-mediated increase in cleaved caspase-3, cleaved caspase-7, and cleaved PARP levels. In contrast, imatinib-mediated induction of $p27^{KIP1}$, an important CDK inhibitor involved in cell cycle transition from G_1 to S [Bloom and Pagano, 2003], was not suppressed by hemin treatment and transfection with Nrf2 siRNA had no influence on $p27^{KIP1}$ level. These results thus suggest that heme suppresses imatinib-induced apoptosis signaling through upregulation of Nrf2 function, which, however, does not involve a change in $p27^{KIP1}$ expression.

Inhibition of Heme Synthesis Increases Imatinib Sensitivity in Imatinib-Resistant Cells

We next examined IC_{50} values of imatinib in imatinib-resistant KCL22/SR and KU812/SR cells, incubated either in the presence or absence of succinylacetone, which is a potent inhibitor of ALA dehydratase, thereby also of heme synthesis [Sassa and Kappas, 1983]. Results showed that the addition of succinylacetone to cells in culture decreased the IC_{50} value of imatinib in the two imatinib-resistant cell lines (Table II). These results therefore also support our conclusion that heme is critically involved in the determination of sensitivity of cells to imatinib.

Hemin Treatment Decreases Cellular Sensitivity to Certain Other Anti-Leukemia Drugs

Next, we examined the effect of hemin on the sensitivity of KCL22 cells to conventional anti-leukemia drugs such as cytarabine, etoposide, hydroxyurea, daunorubicin, idarubicin, doxorubicin, and mitoxantrone. While IC_{50} values of cytarabine, etoposide and hydroxyurea were not altered by hemin treatment, hemin treatment significantly increased IC_{50} values of four anthracyclin anti-leukemia drugs, daunorubicin, idarubicin, doxorubicin, and mitoxantrone (Table III). Addition of ALA also increased IC_{50} values of three of the four anthracyclins (Table III). In contrast, hemin treatment had no effect on IC_{50} values of Rapamycin, LY294001, and U0126, which are small molecules targeting mTOR, PI3-kinase, and MEK1/2, respectively. These results suggest that heme may also be involved in the determination of sensitivity of cells to certain other anti-leukemia reagents, such as anthracyclins.

We also examined the effect of hemin on the sensitivity of BCR/ABL-negative leukemia cell lines U937 and THP-1 to anthracyclins. As shown

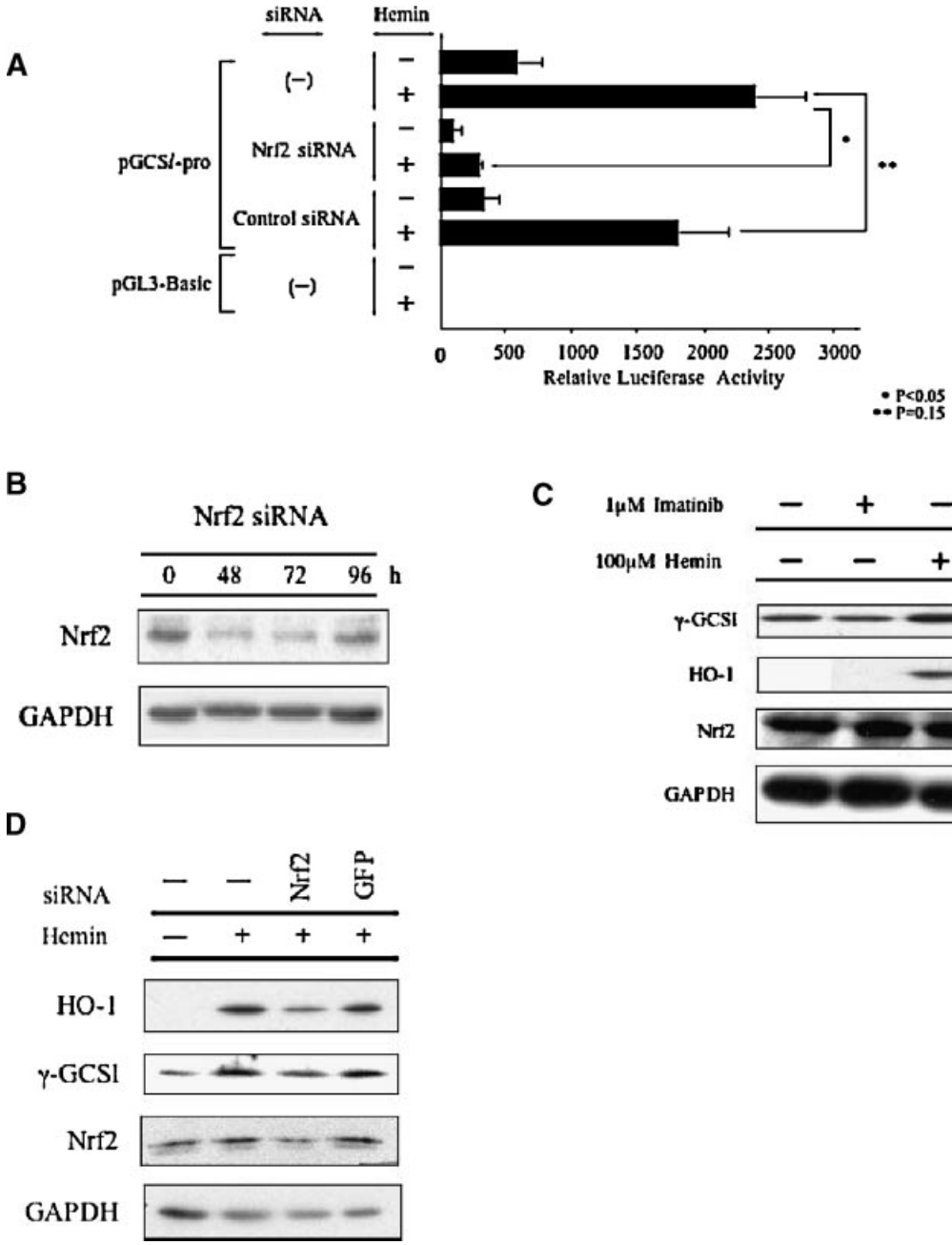

Fig. 3. Hemin treatment increases Nrf2-mediated gene expression. **A**: KCL22 cells were transfected with luciferase reporter pGCS-pro or pGL3-Basic as described in Section ''Materials and Methods.'' Firefly luciferase activity was normalized on the basis of Renilla luciferase activity. Results are expressed as the ratio of firefly luciferase activity of γ-GCS-pro-transfected cells to that in pGL3-basic-transfected cells without addition of hemin. **B**: KCL22 cells were transfected with Nrf2 siRNA as described in Section ''Materials and Methods.'' Cells were harvested and cell lysates were prepared at each time point as indicated in the figure. Immunoblot analysis using human anti-Nrf2 antibody was performed as described in Section ''Materials and Methods.'' Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was used as a control for loading. **C**: Cells were treated with 100 μM hemin, 1 μM imatinib, or with hemin and imatinib for 24 h. Immunoblot analyses using human anti-γ-GCS light subunit, anti-heme oxygenase-1, anti-Nrf2 and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were performed as described in Section ''Materials and Methods.'' **D**: KCL22 cells were transfected with Nrf2 or GFP siRNAs and cultured for 24 h prior to treatment with 100 μM hemin for another 24 h. Immunoblot analyses using human anti-γ-GCS light subunit, anti-heme oxygenase-1, anti-Nrf2 and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were performed as described in Section ''Materials and Methods.''

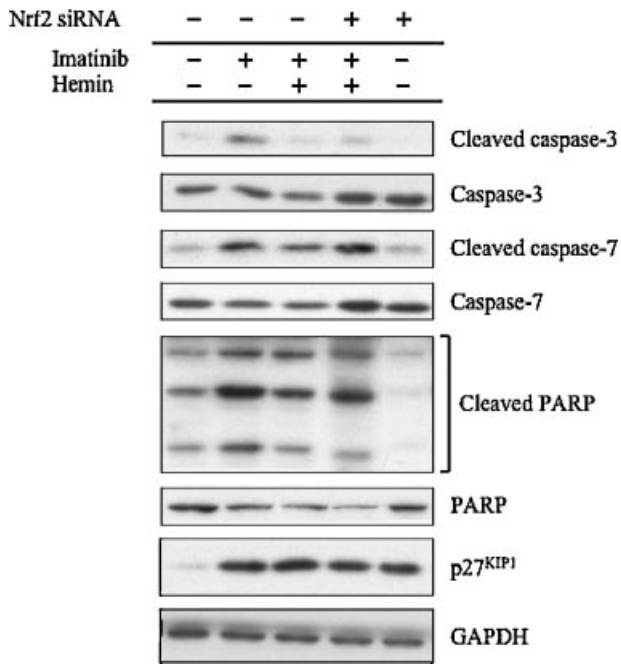

Fig. 4. siRNA-mediated knockdown of Nrf2 restores the hemin-mediated suppression of imatinib sensitivity. KCL22 cells were transfected with Nrf2 siRNA and cultured for 24 h prior to treatment with a combination of 1 μM imatinib and 100 μM hemin for another 24 h. Immunoblot analyses using anti-caspase-3, anti-cleaved caspase-3, anti-caspase-7, anti-cleaved caspase-7, anti-PARP, anti-cleaved PARP, and anti-$p27^{KIP1}$ rabbit polyclonal antibodies were performed as described in Section ''Materials and Methods.'' Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was used as a control for loading.

in Figure 5, addition of 100 μM hemin to cell culture significantly improved anthracyclin-mediated suppression of cell growth. These results strongly suggest that the sensitivity to anthracyclins is also regulated by heme in BCR/ABL-negative cells, as in BCR/ABL-positive cells.

DISCUSSION

The aim of this study was to determine the mechanism of heme-mediated modulation of cellular sensitivity to anti-leukemia drugs such as imatinib and anthracyclins. It is known that hemin exogenously added to cultured cells becomes heme and constitutes the heme moiety of hemoglobin in MEL cells [Granick and Sassa, 1978]. We found that IC_{50} values of imatinib increased following hemin treatment of BCR/ABL-positive KCL22 cells in a dose-dependent manner (Fig. 1A and Table I). Similar to hemin treatment, treatment of cells with ALA, the heme precursor, also increased IC_{50} values of imatinib. Our results also demonstrated that apoptosis induced by imatinib was suppressed following hemin treatment (Fig. 1B). These results suggest that the sensitivity of cells to imatinib may be decreased by an increase in cellular heme concentration. In support of this conclusion, inhibition of heme synthesis by the addition of succinylacetone resulted in a decrease in IC_{50} values of imatinib in imatinib-resistant KCL22/SR and KU812/SR cells

TABLE II. Effect of Succinylacetone on IC_{50} Values of Imatinib in KCL22/SR and KU812/SR Cells

	IC_{50} value (μM)		
Cell line	Control	Succinylacetone	Fold increase
KCL22/SR	2.02 ± 0.05	1.25 ± 0.42	0.62*
KU812/SR	0.75 ± 0.33	0.47 ± 0.20	0.63*

Statistical analysis was carried out for comparison of the data between control cells and succinylacetone-treated cells (*$P < 0.05$). Data are shown as means ± SEM of three independent experiments.

TABLE III. Effects of Hemin on IC_{50} Values of Other Anti-Leukemic Drugs and Small Molecular Compounds

	IC_{50} value		
Reagent	Control	Hemin (fold increase)	ALA (fold increase)
Ara-C	6.65 ± 4.69	8.70 ± 5.88 (1.31)	ND
DNR	17.7 ± 3.05	41.9 ± 2.95 (2.367)*	49.2 ± 19.0 (2.78)*
IDA	12.0 ± 2.49	201.2 ± 74.8 (16.74)*	8.98 ± 2.79 (0.75)
MIT	11.9 ± 2.56	37.7 ± 16.1 (3.2)*	36.1 ± 8.88 (3.07)*
DXR	31.4 ± 5.69	238.3 ± 78.3 (7.59)*	67.1 ± 18.0 (2.14)*
VP-16	2.18 ± 0.97	1.80 ± 1.66 (0.83)	ND
HU	561.6 ± 183.2	824.5 ± 282.4 (1.47)	ND
Rapamycin	4.02 ± 1.07	1.74 ± 0.69 (0.43)	ND
LY-294002	7.08 ± 2.11	5.21 ± 2.47 (0.74)	ND
U0126	8.25 ± 0.63	22.5 ± 15.7 (2.73)	ND

δ-ALA, δ-aminolevulinic acid; Ara-C, cytarabine; DNR, daunorubicin; IDA, idarubicin; MIT, mitoxantrone; DXR, doxorubicin; VP-16, etoposide; HU, hydroxyurea. The units of IC_{50} values are "nM" for Ara-C, DNA, IDR, MIT, DXR, Rapamycin; "μM" for VP-16, LY-294002, U0126; and "mM" for HU. Statistical analysis was carried out for comparison of the data between control cells and hemin or ALA-treated cells (*$P < 0.05$). Data are shown as means ± SEM of three independent experiments. ND, not done.

(Table II). All of these findings thus suggest that heme plays an important role in the determination of the sensitivity of cells to imatinib.

Elucidation of mechanisms for the heme-mediated decrease in imatinib sensitivity is of great interest. The findings in this study demonstrate that hemin treatment significantly increased MARE-mediated γ-GCS light subunit gene promoter transactivity, while transfection of Nrf2 siRNA virtually abolished this effect (Fig. 3A), suggesting that heme modulates MARE-mediated gene expression via regulation of Nrf2 activity. Although changes in the levels of Nrf2/MARE and Bach1/MARE complexes were not directly examined in this study, it is likely that hemin-induced activation of Nrf2 is a result of an increase in the formation of Nrf2/MARE complex, due to decreased DNA binding of Bach1 as reported previously [Shibahara, 2003; Dhakshinamoorthy et al., 2005]. Knockdown of Nrf2 expression by Nrf2 siRNA transfection also abolished the repressive effect of hemin on cellular sensitivity to imatinib. In addition, ascorbic acid treatment, which is known to reduce Nrf2/DNA complex formation, restored cellular sensitivity to imatinib in hemin-treated cells (data not shown) [Tarumoto et al., 2004]. Addition of BSO, a potent inhibitor of γ-GCS, also partially restored the sensitivity to imatinib in hemin-treated cells (data not shown). These findings thus suggest that activation of Nrf2 is critically involved in heme-mediated repression of imatinib sensitivity.

To determine whether the effect of hemin on the suppression of sensitivity to imatinib is specific to KCL22 cells, we examined the effects of imatinib in two other BCR/ABL-positive cell lines, K562 and KU812, both in the presence and absence of hemin. Addition of hemin resulted in a significant increase in IC_{50} values of imatinib (5.3- and 5.1-fold in K562 and KU812 cells, respectively). Since these cells are also known to undergo erythroid cell differentiation by hemin treatment, it is possible that increased heme synthesis as a result of cellular differentiation additionally contributes to the observed increase in IC_{50} values of imatinib.

Our findings thus far clearly demonstrated that increased cellular heme concentration decreases the sensitivity of cells to imatinib. We also examined whether there is a difference in heme concentration in untreated imatinib-resistant and imatinib-sensitive cell lines, using a fluorometric method described previously [Sassa, 1976]. However, there was little difference in the amount of heme among imatinib-resistant cell lines, KCL22/SR, K562/SR, and KU812/SR, and their corresponding imatinib-sensitive KCL22, K562, and KU812 cells (data not shown). It is possible that, if a more sensitive method becomes available, it may uncover a small difference in heme concentration between imatinib-resistant and sensitive cell lines. However, it was not possible to detect any difference using the fluorometric method that is presently the most sensitive assay for heme concentration. It would be also of interest to examine if there may be difference in cellular heme

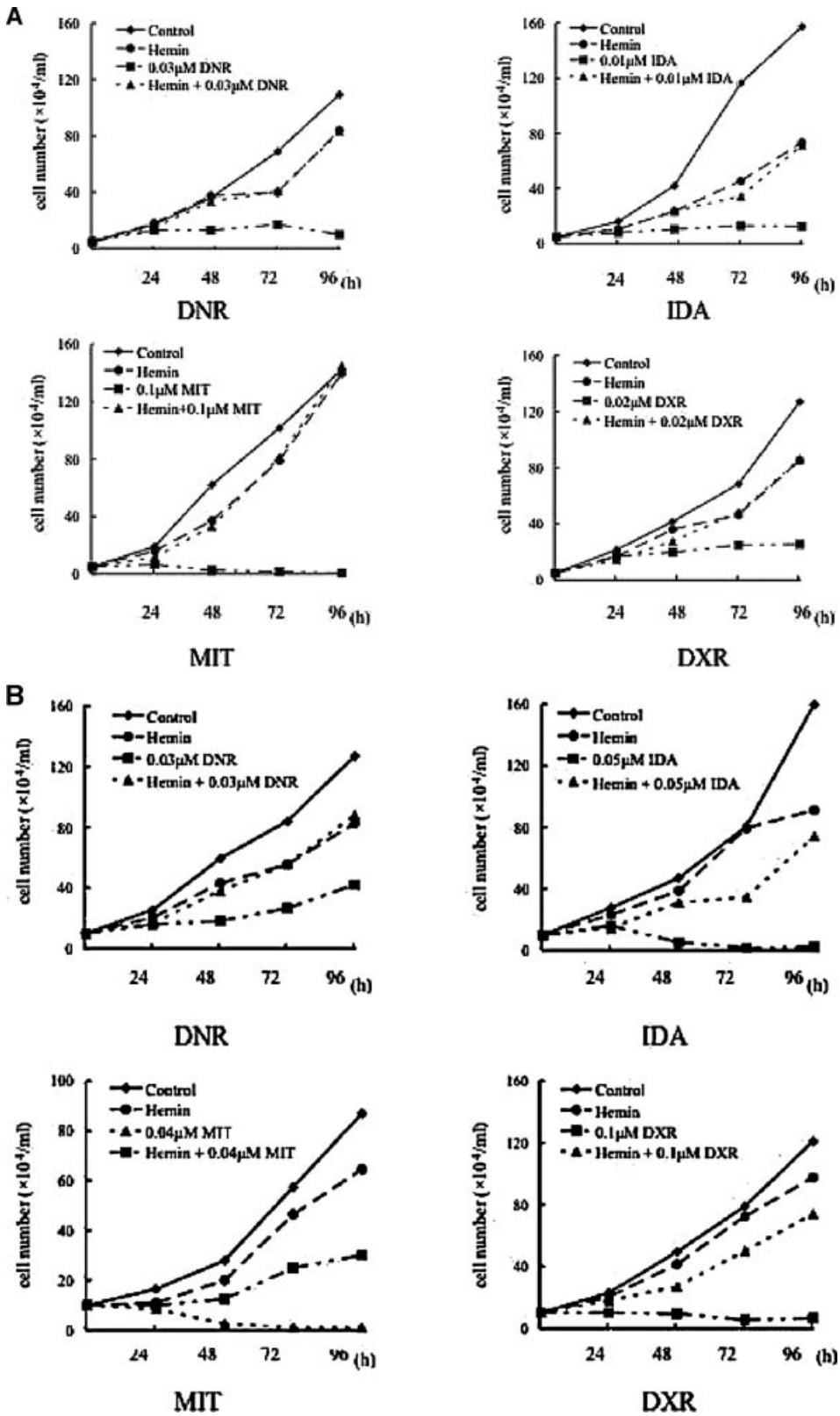

Fig. 5. Effect of hemin on anthracyclin-mediated growth inhibition. U937 (**A**) and THP-1 (**B**) cells were incubated with each anthracyclin at the indicated concentration or additionally with 100 μM hemin. Cells were harvested at various time points as indicated in the figure. The number of viable cells was counted using trypan blue staining.

concentrations between imatinib-sensitive and resistant patients when a more sensitive assay becomes available.

Since our data showed that hemin-mediated suppression of imatinib sensitivity is not due to decreased BCR/ABL kinase activity, the target of imatinib action, it is possible that heme is also involved in the determination of cellular sensitivity to other anti-leukemia drugs. In fact, IC_{50} values of four anthracyclin anti-leukemia drugs in KCL22 cells were increased when hemin was added, and IC_{50} values of the four anthracyclins except for idarubicin were also increased by ALA treatment (Table III). It is not clear why ALA did not influence the sensitivity to idarubicin, but the results for the other three anthracyclins suggest that heme plays an important role in the determination of sensitivity to anthracyclins. Interestingly, hemin restored the inhibition of cell growth mediated by each anthracyclin in BCR/ABL-negative leukemia cell lines, U937 and THP-1 (Fig. 5). These results strongly suggest that heme regulates the sensitivity to anthracyclins, irrespective of the presence of BCR/ABL protein in leukemia cells. It is important to clarify whether Nrf2 is also involved in the heme-mediated suppression of sensitivity of cells to anthracyclins, and such studies are currently under way in our laboratory. In contrast, IC_{50} values of other conventional anti-leukemia agents examined in this study were not increased in the presence of hemin. Furthermore, hemin had no effect on the Ara-C and etoposide-mediated induction of cleaved PARP irrespective of the presence or the absence of Nrf2 siRNA (data not shown). Therefore, it is likely that Nrf2 is not involved in the determination of sensitivity to other conventional anti-leukemia agents such as Ara-C and etoposide, and it is perhaps the reason why hemin does not influence the sensitivity of cells to these agents.

In conclusion, the findings in this study demonstrated that heme is involved in the determination of sensitivity of BCR/ABL-positive leukemia cells to imatinib through regulation of Nrf2 function. The suppressive effect of heme on drug sensitivity is not restricted to imatinib, but is also found for other anthracyclin anti-leukemia agents, and is not influenced by BCR/ABL-positive or -negative nature of cells. These findings suggest that a better understanding of mechanisms involved in the regulation of drug sensitivity will be useful for development of more specific and safer anti-leukemic drugs and that cellular heme concentration can be a potential target for such an endeavor.

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