Biochemical Pharmacology, Vol. 53, pp. 1419–1424, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(96)00894-5

Modulation of Transferrin Receptor Expression by Dexrazoxane (ICRF-187) via Activation of Iron Regulatory Protein

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ABSTRACT. Dexrazoxane (ICRF-187) has recently been demonstrated to reduce cardiac toxicity induced by chemotherapy with anthracyclines, although the reason for this phenomenon has remained obscure thus far. In order to investigate whether ICRF-187 might exert its effects by modulating iron metabolism, we studied the drug's potential to influence the maintenance of iron homeostasis in two human cell lines. We demonstrate that ICRF-187 enhanced the binding affinity of iron regulatory protein (IRP), the central regulatory factor for posttranscriptional iron regulation, to RNA stem loop structures, called iron responsive elements (IRE), in THP-1 myelomonocytic as well as K562 erythroleukemic cells. Increased IRE/IRP interaction was paralleled by an elevation of transferrin receptor (trf-rec) mRNA levels which, according to the well-established mechanism of posttranscriptional iron regulation, was likely due to stabilisation of trf-rec mRNA by IRP. Subsequently, ICRF-187 treatment of cells increased trf-rec surface expression and enhanced cellular iron uptake. All these events, i.e. IRP activation, stabilisation of trf-rec mRNA and increased surface expression of the protein in response to ICRF-187, follow a dose-response relationship. Increased cellular uptake and sequestration of iron in response to ICRF-187 may contribute to the protective activity of ICRF-187 by reducing the iron-anthracycline complex and iron-catalysed generation of hydroxyl radicals via the Haber–Weiss reaction. BIOCHEM PHARMACOL 53;10:1419–1424, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. iron metabolism; transferrin receptor; human monocytes; iron regulatory protein; ICRF-187

Dexrazoxane (ICRF-187; (+)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane) has been demonstrated to exert protective effects against development of anthracycline-induced cardiomyopathy, which is a frequent and cumulative dose-limiting side-effect of anticancer therapy with anthracyclines such as adriamycin or daunorubicin [1–5]. However, the reason for this phenomenon has not been fully elucidated thus far. Besides their DNA-intercalation potential, anthracyclines are powerful inducers of free radical species by generating oxygen radicals either directly or via formation of complexes with ferric iron (for review see 6). The latter molecule can then, after undergoing further internal redox reactions, transfer electrons to molecular oxygen, thereby catalysing the formation of free radicals [7]. Anthracycline-induced formation of radicals causes lipid peroxidation of mitochondrial membranes and endoplasmatic reticulum, suggested to be a major cause of anthracycline-induced cardiotoxicity, since reduced levels of radical scavenging mechanisms appear to be present in the heart as compared to other tissues [6, 8].

It was suggested that ICRF-187 could chelate iron and therefore reduce anthracycline-mediated toxicity due to the fact that the open-ring hydrolysis product of ICRF-187, namely ADR-925, is a cycled analogue of EDTA. In this respect, it was interesting to find that ICRF-187 can recruit iron from the iron storage protein ferritin. Furthermore, intracellular iron chelators such as desferrioxamine were shown to exert protective activity against anthracycline-induced cardiotoxicity comparable to that of ICRF-187 [9–11]. Therefore, this study was designed to investigate the potential effects of ICRF-187 on the regulation of cellular iron metabolism.

Maintenance of cellular iron homeostasis is largely exerted posttranscriptionally by the interaction of specific cytoplasmatic proteins, named iron regulatory protein (IRP)-1 and IRP-2, with RNA stem loop structures, called iron responsive elements (IRE). IREs are present within the 5′ untranslated regions of the mRNAs for the iron storage protein ferritin and the key enzyme of haem biosynthesis, erythroid 5-aminolaevulinate synthase (e-ALAS), and also within the 3′ untranslated region of transferrin receptor (trf-rec) mRNA, the central molecule for iron uptake into cells (for review see 12–14). IRP-1 is a bifunctional protein which can, depending on intracellular iron supply, act

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Abbreviations: trf-rec, transferrin receptor; IRP, iron regulatory protein; IRE, iron responsive element; e-ALAS, erythroid 5-aminolaevulinate synthase; NO, nitric oxide.

Received 5 June 1996; accepted 20 November 1996.

either as a cytoplasmic aconitase or an iron-regulatory protein [15–17]. During cellular iron deprivation states, in the case of enhanced intracellular formation of the labile radical nitric oxide (NO) or during oxidative stress IRE-binding function of IRPs is stimulated [18–23]. Activated IRPs then target IREs at the 5′-untranslated region of ferritin mRNA and e-ALAS mRNA with high affinity, resulting in repression of translation for these proteins [12–14]. Conversely, binding of IRPs to IREs within the 3′ untranslated region of trf-rec mRNA enhances the stability and consequently the expression of this RNA by protecting it from digestion by a not yet identified RNase, which promotes iron uptake into cells [12–14]. Increased intracellular concentrations of low molecular weight iron then decrease the IRE-binding function of IRPs, which then lose their high affinity towards IREs. This results in increased ferritin and e-ALAS translation and reduced trf-rec mRNA stability, which leads overall to iron storage and iron consumption as well as low iron uptake [12–14, 18–23].

Based on our supposition concerning a putative biochemical function for ICRF-187, we investigated the effects of this substance on activation of IRPs, trf-rec mRNA stability and expression and cellular iron uptake in the human erythroleukemic cell line K562 and the human myelomonocytic cell line THP-1, both of which are frequently used for investigating regulation of iron metabolism and/or macrophage function, respectively.

MATERIALS AND METHODS

Cell Culture Techniques

The human monocytic cell line THP-1 and the human erythroleukemic cell line K562 were both obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin at 37°C in humidified air containing 5% CO_2. Cells were seeded at a density of 0.5×10^6/mL, and 50 μM ferric nitrate nonahydrate [Fe(3+)] (applied as $Fe(NO_3)_3 \cdot 9H_2O$ from Sigma, Munich, Germany), 100 μM desferrioxamine (Sigma, Munich, Germany) or 10 μg/mL to 1 mg/mL (corresponding to 25 μM to 2.5 mM) ICRF-187 hydrochloride-salt (a gift from Laevosan, Austria) were then added to the culture medium. After incubation for 24 hr, the incubated cells were harvested, washed twice with phosphate-buffered saline and subjected to further procedure as indicated below.

Gel-retardation Assay

K562 and THP-1 cells were grown in RPMI plus additives and treated as described above. After harvesting and washing, detergent cell extracts were prepared as described previously [19]. A [^{32}P]-labelled human ferritin H-chain IRE probe was generated by an *in vitro* transcription procedure and purified by gel electrophoresis (15% acrylamide/bisacrylamide (20:1), 6 M urea) following probe elution, phenol/chloroform extraction and ethanol precipitation as described [24]. Approx. 12000 cpm of this transcript were incubated with 20 μg protein of each cellular extract at room temperature. After 20 min, 3 mg/mL heparin were added for ten min, and analysis of RNA/protein complexes was carried out by non-denaturing gel electrophoresis and subsequent autoradiography as described [18].

RNA Extraction and Northern Blot Analysis

Cells were stimulated for 24 hr as described above. Preparation of total RNA was then carried out by acid guanidinium thiocyanate-phenol-chloroform extraction according to [25]. Ten μg of total RNA were separated on 1% agarose/2.2 M formaldehyde gels and RNA was blotted onto Duralon-UV membranes (Stratagene, LA Jolla, CA, USA). After UV-crosslinking and prehybridisation for 6 to 8 hr at 65°C, blots were hybridised overnight with 10^6 cpm/mL of [α-^{32}P]dCTP-radiolabelled cDNA plasmid probes at 65°C. The hybridisation solution contained 3 × SSC, 0.1% SDS, 0.1% sodiumpyrophosphate, 10% dextran sulphate, 10 × Denhardt's solution (0.2% Ficoll 400, 0.2% polyvinylpyrrolidone, 0.2% BSA), and 1 mg/mL of denaturated salmon sperm DNA. Blots were washed subsequently with 2 × SSC/0.5% SDS (twice for 30 min) and with 0.1 × SSC/0.5% SDS (twice for 30 min) at 65°C. Filters were exposed for up to 4 days to XRP-5 x-ray films (Kodak, X-OMAT RP, Sigma) with intensifying screens at −80°C. Human transferrin receptor cDNA was kindly provided by Dr. M.W. Hentze (EMBL, Heidelberg). For northern hybridisation, the 800 bp AccI insert was used. Chicken β-actin cDNA (in pBR322; 1.9 kb HindIII/HindIII insert) was a generous gift from Dr. D. W. Cleveland. Probes were labelled with [α-^{32}P]dCTP (DuPont New England Nuclear, Boston, MA, U.S.A.) using the oligoprimer procedure [26]. Autoradiographs were densitometrically scanned using the Bio-Profil system for image analysis (Vilber Lourmat, Marne La Vallée, France).

Determination of Transferrin Receptor Expression on Cell Surface

Expression of human trf-rec was estimated by using a rabbit anti-human CD 71 monoclonal antibody (obtained from Boehringer Mannheim, Germany). For isotype control, a mouse IgG2a monoclonal antibody anti CD-10 (Becton-Dickinson, Mountain View, CA, USA) was used. Both antibodies were applied as purified immunoglobulin (0.5 μg in 20 μL of PBS containing 0.1% azide). Cells were seeded at a density of 10^6 cells/mL in 24-well plates and supplemented and stimulated as described above for 48 hr. After harvesting by scraping, cells were washed and resuspended in 50 μL of DMEM containing 2% FCS and 0.1% azide and incubated with the appropriate antibody for 30 min on ice. For reagent control, the first step was carried out only with

culture medium. Incubation was stopped by addition of 1 mL of cold DMEM/2%FCS/0.1% azide, and after washing twice, cells were counterstained with fluorescein isothiocyanate-conjugated anti-mouse IgG serum diluted 1/100 in DMEM/2%FCS/0.1% azide for 40 min on ice. Stained cells were analysed on a FACStar flow cytometer (Becton Dickinson, Mountain View, CA, USA). Fluorescence intensities of 5×10^3 cells were measured for each determination. Data are expressed as mean fluorescence channel in relation to isotype and reagent control.

Iron Uptake Studies

Cells were incubated for six hours in serum-free medium (RPMI 1640 with 1 mg/mL HSA) with appropriate additives, and then incubated with [^{59}Fe]-labelled transferrin for two hr as described [27]. Radioactivity in cells and supernatants was determined, and uptake expressed as ng Fe taken up per 10^6 cells per hr.

Protein Determination

Protein concentration of cell lysates was estimated according to Bradford [28] using the protein dye reagent from BioRad (Richmond, California, U.S.A.) and bovine serum albumin as a standard.

Statistical Analysis

Calculation of statistical significance was carried out by Student's *t*-test. Only P values < 0.05 were considered significant.

RESULTS

ICRF-187 Enhances IRE-binding Affinity of IRP

In order to investigate a possible effect of ICRF-187 on iron regulation, we first performed gel-retardation assays to obtain an estimate for alterations in IRE-binding activity of IRP. In K562 as well as in THP-1 cells, only one IRE/IRP complex could be detected, corresponding to human IRP-1. As previously described [18, 19, 22], treatment of cells with ferric iron nitrate (50 μM) for 24 hr caused a decrease in IRP-1-binding activity as compared to untreated control cells, while addition of the intracellular iron chelator desferrioxamine (100 μM) resulted in an up to three-fold increase in IRE/IRP interaction as compared to the control (Fig. 1). When cells were exposed to increasing concentrations of ICRF-187, ranging from 10 μg/mL to 1 mg/mL (corresponding to final concentrations between 25 μM to 2.5 mM), a growing stimulation of high affinity binding of IRP to IRE could be observed (Fig. 1). The observed alterations in IRP-binding affinity after iron perturbations or ICRF-187 treatment were evident in both human erythroleukemic cells (K562, Fig. 1) and human myelomonocytic cells (THP-1, results not shown).

Addition of 2-mercaptoethanol to cellular extracts (final concentration 2%), a procedure known to fully activate IRE-binding of IRP in vitro [19], caused maximum high affinity binding of IRP to IREs with either treatment (Fig. 1). This indicates that changes in IRP-1 activity upon iron perturbations or treatment with ICRF-187 occur posttranslationally.

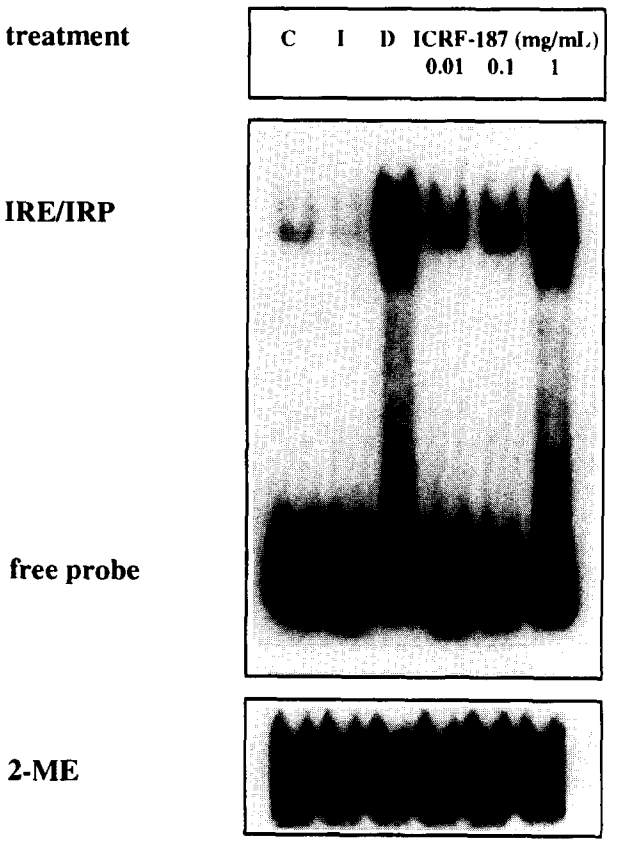

FIG. 1. Activation of IRP by ICRF-187. K562 cells were treated with desferrioxamine (D; 100 μM corresponding to 66 μg/mL), ferric iron nitrate (I; 50 μM), ICRF-187 (10 μg/mL to 1 mg/mL corresponding to 25 μM to 2.5 mM, respectively), or left untreated (control; C). After 24 hr detergent cell extracts were prepared and analysed for the IRE-binding activity of IRP by electromobility shift assay using a [^{32}P]-labelled IRE probe. IRE/IRP complexes and unbound IRE probe are shown in the upper panel, while in the lower panel (extracts plus 2% mercaptoethanol) only IRE/IRP complexes are depicted. One of four similar experiments is shown. Gel-retardation assays with extracts from THP-1 gave results comparable to those shown for K562 (data not shown).

Upregulation of trf-rec mRNA by ICRF-187

We then tested whether the stimulation of the IRE-binding function of IRP-1 by ICRF-187 would result in modulation of trf-rec mRNA concentrations, since the expression of this protein is subjected to posttranscriptional regulation by IRPs [14, 20]. As is evident from Fig. 2, cytoplasmic concentrations of trf-rec mRNA are increased in K562 cells after treatment with desferrioxamine, which is due to stabilisation of trf-rec mRNA following IRE/IRP interaction, as previously demonstrated [20]. Accordingly, trf-rec

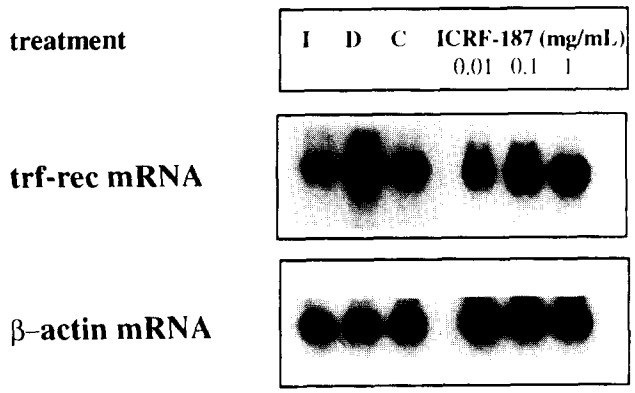

FIG. 2. ICRF-187 increases trf-rec mRNA expression. K562 cells were treated as described in the legend to Fig. 1. After 24 hr total RNA was prepared, and 10 μg were subjected to Northern blot analysis. One of three similar experiments is shown. Northern blot analysis of THP-1 cells provided the same pattern of trf-rec mRNA concentrations depending as pictured for K562 cells for either treatment (data not shown).

mRNA levels are reduced following iron challenge, a procedure which also decreases the IRE-binding activity of IRP-1 (Fig. 1 and 2). Densitometric scanning of Northern blots clearly demonstrated a growing increase in trf-rec mRNA levels upon supplementation of increasing dosages of ICRF-187 (1.2 ± 0.4 [increase of the trf-rec/β-actin mRNA ratio relative to the control where the ratio is set at "1" corresponding to "100%"] for 10 μg/mL (25 μM) of ICRF-187 (N = 3), 1.8 ± 0.3 for 100 μg/mL (250 μM) ICRF-187 (N = 3), and 2.3 ± 0.4 for 1 mg/mL [2.5 mM] of ICRF-187 (N = 3), respectively; $P > 0.05$ for 10 μg/ml ICRF-187; $P < 0.01$ for 100 μg/ml and 1 mg/ml of ICRF-187).

The changes in trf-rec mRNA levels are specific, since β-actin mRNA levels were not altered by either treatment (Fig. 2, lower panel).

The effects observed for ICRF-187 on trf-rec mRNA concentrations were consistent in both cell lines investigated, although only the results for K562 are shown here (Fig. 2). Treatment of these cell lines (K562 and THP-1) with the indicated dosages of ICRF-187 for 24 hr did not result in a decreased cellular viability in comparison to untreated control cells as checked by trypan blue exclusion (details not shown).

ICRF-187 Increases trf-rec Surface Expression

As shown in Table 1, ICRF-187 significantly increased trf-rec expression on the surface of both human erythroleukemic (K562) and human myelomonocytic cells (THP-1) after an incubation period of 48 hr, which indicates that the increased concentrations of trf-rec mRNA observed after ICRF-187 treatment cause an enhanced cell surface expression of trf-rec. As with the activation of IRP-1 and the increase in cytoplasmic trf-rec mRNA levels, the effects of ICRF-187 on surface expression were dose-dependent. Administration of ICRF-187 at a dosage 0.1 mg/mL [250 μM] resulted in comparable amounts of trf-rec surface expression as observed after administration of desferrioxamine (100 μM, corresponding to 66 μg/mL). This indicates that at equal concentrations desferrioxamine appears to be more effective in regulating trf-rec expression than ICRF-187. Conversely, iron treatment resulted in a slight, although not significant decrease in trf-rec expression as compared to untreated control cells.

TABLE 1. Stimulation of trf-rec expression on K562 human erythroleukemic and THP-1 human monocytic cells by ICRF-187

	Mean channel log fluorescence	
Treatment	K562 cells	THP-1 cells
Control	23.2 ± 4.8	13.3 ± 2.4
Iron	19.8 ± 5.9‡	10.7 ± 2.5‡
Desferrioxamine	61.2 ± 19.6*	37.2 ± 12.9*
ICRF-187 (10 μg/mL)	35.4 ± 12.6‡	26.8 ± 8.7†
ICRF-187 (100 μg/mL)	58.7 ± 16.2*	49.4 ± 15.4*
ICRF-187 (1 mg/mL)	124.0 ± 21.6*	72.6 ± 12.0*

K562 or THP-1 cells were treated with desferrioxamine (100 μM corresponding to 66 μg/mL), ferric iron nitrate (50 μM), ICRF-187 (10 μg/mL to 1 mg/mL corresponding to 25 μM to 2.5 mM, respectively), or left untreated (control). After 48 hr, cells were washed and 1×10^6 cells were subjected to immunofluorescence staining for surface trf-rec as described in "Materials and Methods". Data are expressed as mean channel log fluorescence minus fluorescence of the isotype and the reagent control for three different experiments performed in triplicate (means ± SD). Statistical differences in relation to the control were calculated by means of Student's *t*-test. *$P < 0.01$; †$P < 0.05$, ‡$P > 0.05$, not significant.

Stimulation of Cellular Iron Uptake by ICRF-187

Incubation of K562 cells with ICRF-187 (100 μg/mL corresponding to a final concentration of 250 μM) resulted in a significant ($P < 0.025$) increase in transferrin-mediated iron uptake into K562 cells (0.31 ± 0.05, N = 4 vs 0.23 ± 0.05 ng Fe/10^6 cells per hr for controls) as was also observed for cells treated with desferrioxamine (100 μM corresponding to 66 μg/mL; 0.33 ± 0.06 ng Fe/10^6 cells/hr; $P < 0.025$ as compared to the control; $P > 0.05$ as compared to ICRF-187-treated cells). Although the increase in iron uptake with desferrioxamine and ICRF-187 was relatively small, it was consistent and reproducibly observed.

DISCUSSION

We were able to demonstrate that ICRF-187 influences posttranscriptional regulation of iron metabolism via activation of IRP-1, thus causing a subsequent increase in trf-rec mRNA concentrations, trf-rec expression and iron uptake. These metabolic changes were observed in two different cell lines, thus pointing to the generality of this mechanism at least in human cells. The effects of ICRF-187 were comparable to those of desferrioxamine, which indicates that ICRF-187 may act in a similar fashion [29]. Therefore, according to the well-established mechanism of posttranscriptional iron regulation by intracellular iron

perturbations [12–14, 20], the up-regulation of trf-rec mRNA levels in response to ICRF-187 treatment should be due to stabilisation of this mRNA following IRP/IRE interaction within its 3′ untranslated region. The effect of ICRF-187 on IRP-1 is most likely due to one of two mechanisms: direct interaction with the central iron-sulphur cluster of the protein, thus causing an allosteric conformational change which enhances the IRE-binding affinity of IRP-1 while its cis-aconitase function is reduced [12–17]; alternatively, ICRF-187 may reduce the amount of metabolically available iron in the cell, thereby influencing the proposed turnover of the central iron-sulphur cluster of IRP-1. However, ICRF-187-induced IRP-1 activation enhances trf-rec expression and iron uptake into cells. Elevated intracellular concentrations of low molecular weight iron may then cause deactivation of IRP-1, as described above, which promotes ferritin translation and iron storage [12–14].

Iron has been shown to be of central importance for radical formation via its catalytic function for the synthesis of hydroxyl radical in the so called *Haber–Weiss* reaction (for review see 30, 31). Most interestingly, iron sequestration by macrophages has recently been detected as a powerful mechanism to reduce extracellular formation of hydroxyl radicals via the *Haber–Weiss* reaction, thus protecting local tissue from free radical damage via iron-catalysed oxidants [32]. According to the data presented herein, it remains to be seen to what extent stimulation of transferrin-mediated iron uptake and sequestration by ICRF-187 may reduce toxic radical formation by the catalytic action of iron and iron/anthracycline complexes, thus contributing to the protective effects of the drug.

Nevertheless, one would assume that chelation and uptake of iron after ICRF-187 administration reduces the toxic potential of anthracyclines against tumour cells, which should lead to a decreased response rate and reduced disease-free survival in such patients. Interestingly, ICRF-187 has been proven to exert an anti-tumour potential by itself, which has mainly been attributed to the fact that it is a potent inhibitor of DNA topoisomerase II [33, 34]. However, ICRF-187 is converted intracellularly to ADR-925 with a $T_{1/2}$ of 28 hr [35]. While the latter substance has been proven to act as a potent metal chelator [9], it does not have an inhibitory effect towards DNA topoisomerase II. Therefore, it would also appear reasonable that alternative mechanisms may contribute to the anti-tumour potential of ICRF-187. Future studies will be needed to clarify if ICRF-187 (in a comparable fashion as described for desferrioxamine) may favourably modulate cell-mediated immune effector function: it has been reported that iron-loaded macrophages lose their ability to kill intracellular pathogens via interferon-gamma (IFN-γ) mediated pathways, while iron chelation by desferrioxamine enhances the cytotoxic effector potential of macrophages involving the formation of NO [36–38]. Moreover, it will be most interesting to see whether or not activation of IRP-1 by ICRF-187, as shown herein, may contribute to the anti-tumour potential of the drug by translational inhibition of mitochondrial aconitase [E.C. 4.2.1.3.] expression via IRE/IRP interaction within the 5′-untranslated region of its mRNA [12–17, 39], or whether direct interaction of ICRF-187 with other critical iron-sulphur enzymes such as ribonucleotide reductase may account for an anti-proliferative effect in a similar fashion as previously described for desferrioxamine [40].

The concurrent protective and potential cytopathic effects of a drug such as ICRF-187 may require further intensive studies in order to estimate the ultimate clinical risk-benefit ratio as well as putative new therapeutic targets for this substance, e.g. as a supportive drug for treatment of infections as has already been shown for desferrioxamine [41].

The authors would like to thank Dr. Tracey Houston for support in performing the iron uptake studies. The support of the Austrian research fund "Zur Förderung der Wissenschaftlichen Forschung", project 10603, is also gratefully acknowledged.

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