Effects of Cyclosporin A on Erythropoietin Production by the Human Hep3B Hepatoma Cell Line

By Alessandro M. Vannucchi, Alberto Grossi, Alberto Bosi, Daniela Rafanelli, Marinella Statello, Stefano Guidi, Riccardo Saccardi, and Pierluigi Rossi-Ferrini

There is evidence that the inadequate erythropoietin (Epo) production observed in patients undergoing allogeneic bone marrow transplantation (BMT) might be ascribed to an inhibitory effect caused by the immunosuppressive drug cyclosporin A (CsA). In this in vitro study, we have evaluated the effects of CsA on the release of Epo in the culture medium by the human Hep3B hepatoma cell line. In cultures incubated with both CsA and the nonimmunosuppressive CsA analog MeAla-6, but not with the CsA-unrelated immunosuppressive agent FK-506, the levels of Epo in the medium were significantly reduced in comparison with controls, at concentrations (0.01 to 1.6 μmol/L) not affecting total protein synthetic rate nor the constitutive secretion of α-fetoprotein. Hep3B cells were found to contain a CsA-binding molecule, with an M, of 18 Kd, as assessed by high performance liquid chromatography (HPLC) and ligand-blotting analysis. CsA did not affect the expression of the Epo gene, as judged by Northern blot analysis, but caused a significant amount of Epo to remain unsecreted within the cells; almost all (97%) of total of the intracellular Epo was associated with the plasma membrane subcellular fraction. We conclude that: (1) CsA is able to inhibit Epo release in vitro by Hep3B cells, further supporting the hypothesis that the drug might have a role in the inappropriately low Epo levels observed in BMT patients; (2) the inhibitory effect appears to be specific and not caused by a general impairment of protein synthesis and/or secretion; and (3) the reduced Epo levels found in the medium of CsA-treated Hep3B cultures are supposed to be the consequence of an inability of the cells to correctly process Epo molecules for the secretory pathway.

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streptomycin (100 μg/mL), and 10% heat-inactivated fetal calf serum (FCS; Boehringer Mannheim GmbH, Germany). Cells were maintained in a fully humidified, 5% CO2 atmosphere at 37°C, and the culture medium was changed every other day during the logarithmic growth. Stock solutions of CsA and MeAla-6 (a synthetic analog of CsA, modified at the 6 position) at 1 mg/mL were prepared by dissolving the drugs in 10% vol/vol ethanol, 0.2% vol/vol Tween 80 (Boehringer Mannheim) in IMEM. FK-506 was dissolved at 1 mg/mL in 10% vol/vol ethanol in IMEM. From these stock solutions, maintained frozen in aliquots at −70°C, working dilutions in IMEM were freshly prepared. In preliminary experiments, we observed that the addition of the buffers used for dissolving the drugs to mock cultures had no effects on the levels of Epo, when compared with control cultures. Starting from the day before experiment, nearly confluent cultures were fed with a serum-free medium, consisting of IMEM, 1% Nutridoma-SP (Boehringer Mannheim), L-glutamine (0.29 mg/mL), plus penicillin-streptomycin. Epo production by Hep3B cells was routinely induced by incubating cultures with 50 μmol/L cobalt chloride (CoCl2) for 24 hours; in some experiments, cells were also stimulated with 300 μmol/L nickel chloride (NiCl2) or with a low (1%) oxygen tension atmosphere. At the end of the incubation period, supernatants were harvested, clarified by centrifugation, and stored frozen at −20°C until assayed.

Epo levels in supernatants and cell lysates were determined with an enzyme-linked immunosassay kit (Clinigen; Amgen Diagn, Thousand Oaks, CA); each sample was assayed in duplicated using at least two dilutions. Epo levels were normalized to the amount of total cellular protein in cell pellets (Bradford's dye test19), and expressed as μg/mg cell protein. α-fetoprotein (α-FP) levels were measured with a radioimmunoassay kit (Cis Diagnostici, Vercelli, Italy), and expressed as ng/mg cell protein.

Characterization of Hep3B cytosolic CsA-binding protein(s). To prepare cytosolic proteins from Hep3B cells, about 5 × 106 unstimulated cells were collected by mild trypsinization, washed three times with calcium- and magnesium-free phosphate buffered saline (PBS), and either used immediately or stored frozen as a cell pellet at −70°C. Cytosolic protein extraction was performed by homogenizing cells with a ground-glass homogenizer in ice-cold 10 mmol/L Tris-HCl, pH 7.2, containing 150 mmol/L KCl, 0.1% sodium azide, and 1 mmol/L phenylmethylsulphonyl fluoride (Tris-KCl-PMSF buffer), at a 1:4 cell-buffer ratio. 2-mercaptoethanol (2-ME) was then added to give a final concentration of 5 mmol/L, and supernatants from crude homogenates spun at 100,000g for 1 hour (Beckman TL-100 Ultracentrifuge; Beckman Instruments, Palo Alto, CA) were saved. Protein concentration in the supernatant was then determined, and the samples either used immediately for binding assay or stored at −70°C in aliquots.

The binding of [3H]-CsA ([methB-3H] cyclosporin A, specific activity 10.0 Ci/mmol; Amersham Int plc, Buckinghamshire, England) to cytosolic proteins was performed as described.20 The binding buffer consisted of 20 mmol/L Tris-HCl, pH 7.2, containing 5 mmol/L 2-ME, 7.5% FCS, and 0.1% sodium azide. One hundred μL-volumes of cytosolic proteins, of different protein content, were mixed with 50 μL of [3H]-CsA to give 0.5 mmol/L of labeled ligand, in the presence (nonspecific binding) or absence (total binding) of a 200-fold molar excess of unlabeled CsA. The mixture was incubated for 30 minutes at room temperature with occasional agitation, then kept in ice. Free-labeled ligand was separated from the one bound to receptor(s) by loading the sample on minicolumns (total volume, 3 mL) of LH-20 resin (Pharmacia P-L Biochem Inc, Milwaukee, WI); in these experimental conditions, free [3H]-CsA is significantly retarded in the column, and separated from the protein-containing void volume. The fraction corresponding to the void volume was collected and radioactivity counted in a liquid scintillator. Specific binding was calculated by the difference between total and nonspecific binding.

Size-exclusion high performance liquid chromatography (HPLC) was performed with an LKB apparatus (Pharmacia LKB Biotechnology, Uppsala, Sweden) equipped with a TSK-G 3000 SW column. About 1 mL of cytosolic protein extract was concentrated to 0.2 mL with a Centricon 10 microcentrator (Amicon-Grace, Beverly, MA), 0.45 μm filtered, and injected; the column was developed with 20 mmol/L K2HPO4, pH 7.2, at 1.0 mL/min. Fractions of 0.25 mL were collected, and immediately added with 2-ME to give 5 mmol/L final concentration; each fraction was then processed for CsA binding with the LH-20 assay.

The identification of CsA-binding protein(s) by ligand blotting was performed by running a concentrated cytosolic protein sample on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); the sample was simply diluted with unreducing SDS-sample buffer, but not boiled. Separated proteins were electrophoretically transferred to nitrocellulose filters (Bio-Rad, Richmond, CA), and the blot incubated in a blocking buffer, consisting of 25 mmol/L Tris-HCl, pH 7.4, 140 mmol/L NaCl, 4% bovine serum albumin (BSA), and 1% nonfat dried milk. Thereafter, the nitrocellulose sheet was incubated overnight at room temperature with 5 mmol/L [3H]-CsA, in the presence or absence of a 500-fold excess of unlabeled CsA, and then extensively washed. Fluorography-treated gels were exposed at −70°C for 7 to 10 days.

Northern blot analysis. The probe for human Epo was a 40-mer oligonucleotide, whose sequence is derived from the exon 2 of the Epo gene (Oncogene Science, Manhasset, NY). A 7-mer oligonucleotide probe for human β-actin was purchased from Clontech Lab (Palo Alto, CA). Both oligonucleotides were 5'-labeled with [γ-32P] adenosine triphosphate (ATP) (specific activity 6,000 Ci/mmol; NEN-DuPont, GmbH, Dreieich, Germany) and T4-polynucleotide kinase to a specific activity of 3.5 to 5.0 × 107 cpm/pmol.

Total RNA was prepared by cultured cells as described.21 The RNA was denatured by formaldehyde-formamide, and subjected to
electrophoresis in 1% agarose gels in 2.2 mol/L formaldehyde gel running buffer. The transfer of denatured RNA to Hybond-N filters (Amersham) was performed by standard procedures in 10× SSC (saline-sodium citrate buffer). Prehybridization was performed at 42°C for 6 hr with prehybridization buffer (6× SSPE [saline-sodium phosphate–EDTA], 50% formamide, 0.1% SDS, 10% Denhardt solution, 100 μg/mL denatured salmon sperm DNA). Hybridization was performed at 42°C for 16 hr with hybridization buffer (6× SSPE, 50% formamide, 0.1% SDS). The final washing was in 1× SSC at 60°C. Autoradiography was performed with two intensifying screens at −70°C with Hyperfilm-MP (Amersham) for 1 to 3 days.

Intracellular Epo total content and distribution. For the determination of total intracellular Epo content, trypsinized cells were washed twice with PBS, and then dissolved in Tris-KCl–PMSF buffer containing 0.2% Triton X-100 (BDH Chem, Poole, England) for 30 minutes on ice. The sample was centrifuged at 1,000g for 10 minutes at 4°C to precipitate insoluble material, and the clear supernatant was used for Epo determination. In experiments where the intracellular distribution of Epo was determined, trypsinized cells were washed twice, and finally resuspended in Tris-KCl–PMSF containing 0.25 mol/L sucrose. Cell lysis was obtained by forcing the cells through a 27-gauge needle for six times, on ice; the loss of cellular integrity was confirmed by phase-contrast microscopy. Crude nuclei were prepared at 1,000g for 10 minutes, the supernatant collected and spun down at 100,000g for 60 minutes at 4°C. The final supernatant (soluble) was saved, while the membrane pellet was dissolved on ice in Tris-KCl–PMSF buffer containing 0.2% Triton X-100 to yield the membrane-associated Epo fraction.

### Table 1. Effects of CsA on Total Cellular Protein Synthetic Rate

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>[3H]-Aminoacids Incorporated (cpm × 10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>77 ± 16</td>
</tr>
<tr>
<td>CsA (μmol/L)</td>
<td></td>
</tr>
<tr>
<td>1.6</td>
<td>75 ± 19</td>
</tr>
<tr>
<td>0.8</td>
<td>81 ± 25</td>
</tr>
<tr>
<td>0.1</td>
<td>87 ± 27</td>
</tr>
<tr>
<td>0.01</td>
<td>75 ± 23</td>
</tr>
</tbody>
</table>

Hep3B cells were incubated for 16 hours in media containing 50 μmol/L cobalt chloride, in the presence or absence of CsA, and then pulsed for 2 hours with 15 μCi of a mixture of [3H]-aminoacids. Cellular proteins were precipitated with TCA and counted in a beta counter. Results are expressed as the mean ± SD from two experiments.

### Table 2. Effects of CsA and MeAla-6 on the Level of α-FP in the Culture Medium of Hep3B Cells

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>α-FP (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>445 ± 56</td>
</tr>
<tr>
<td>CsA (μmol/L)</td>
<td></td>
</tr>
<tr>
<td>1.6</td>
<td>425 ± 39</td>
</tr>
<tr>
<td>0.8</td>
<td>437 ± 45</td>
</tr>
<tr>
<td>0.1</td>
<td>427 ± 27</td>
</tr>
<tr>
<td>0.01</td>
<td>455 ± 33</td>
</tr>
<tr>
<td>MeAla-6 (μmol/L)</td>
<td></td>
</tr>
<tr>
<td>1.6</td>
<td>445 ± 35</td>
</tr>
<tr>
<td>0.8</td>
<td>438 ± 18</td>
</tr>
<tr>
<td>0.1</td>
<td>416 ± 69</td>
</tr>
<tr>
<td>0.01</td>
<td>445 ± 30</td>
</tr>
</tbody>
</table>

Hep3B cells were incubated in media containing 50 μmol/L cobalt chloride, in the presence or absence of CsA and MeAla-6, and the amount of α-FP was measured 24 hours later. Results are the mean ± SD from two experiments.

In nearly confluent, unstimulated Hep3B cells maintained for 16 to 24 hours under serum-free conditions, Epo was barely detected in the medium (4.0 ± 3.5 mU/mg protein). On the other hand, cultures stimulated with CoCl₂ (50 μmol/L) released high amounts of Epo in the medium, up to 255 ± 40 mU/mg protein (Fig 1). Comparable results were obtained when cells were incubated in an atmosphere containing 1% O₂ (305 ± 95 mU/mg), while stimulation with 300 μmol/L NiCl₂ proved to be less efficient (190 ± 75 mU/mg). Therefore, owing to its simplicity and reproducibility, we have routinely used CoCl₂ for inducing Epo production by Hep3B cells in all subsequent experiments. However, in preliminary experiments not reported in detail, similar effects of CsA on Epo production were observed when Hep3B cells were stimulated with either hypoxia or NiCl₂.

### RESULTS

In nearly confluent, unstimulated Hep3B cells maintained for 16 to 24 hours under serum-free conditions, Epo was barely detected in the medium (4.0 ± 3.5 mU/mg protein). On the other hand, cultures stimulated with CoCl₂ (50 μmol/L) released high amounts of Epo in the medium, up to 255 ± 40 mU/mg protein (Fig 1). Comparable results were obtained when cells were incubated in an atmosphere containing 1% O₂ (305 ± 95 mU/mg), while stimulation with 300 μmol/L NiCl₂ proved to be less efficient (190 ± 75 mU/mg). Therefore, owing to its simplicity and reproducibility, we have routinely used CoCl₂ for inducing Epo production by Hep3B cells in all subsequent experiments. However, in preliminary experiments not reported in detail, similar effects of CsA on Epo production were observed when Hep3B cells were stimulated with either hypoxia or NiCl₂.

Cultures incubated with CsA (in the range of 0.01 to 1.6 μmol/L) showed a dose-dependent reduction in the amount of Epo released into the culture medium after CoCl₂ stimulation (Fig 1). At CsA concentrations of 0.4 μmol/L, about half of the controls' Epo was found in the medium, and decreased further up to about 30% of controls (78 ± 15 mU/mg) at 1.6 μmol/L. The inhibitory effect was not caused by a reduction in the total rate of protein synthesis by Hep3B cells; in fact, the amount of tritiated amino acids incorporated in trichloroacetic acid (TCA) precipitates was virtually comparable in control and CsA-treated cultures (Table 1). Moreover, concentrations of CsA up to 2.0 μmol/L were found not to be toxic to the cells, because no significant modification of total cell number per plate, nor of the
percentage of Trypan-blue$^+$ cells, was observed (data not shown in detail). Finally, to evaluate the specificity of the inhibitory effects of CsA on Epo, the amount of a-FP, a constitutively secreted protein by Hep3B cells, was also measured in culture media. As shown in Table 2, the levels of this protein were not significantly modified by CsA.

We next tested whether the nonimmunosuppressive, stable CsA analog MeAla-6 had similar effects as the parental molecule. As reported in Fig 1, dose-dependently reduced Epo levels were found in cultures incubated with MeAla-6 (0.01 to 1.6 µmol/L), although on a molar basis the analog proved to be less efficient than CsA, especially at the lower concentrations. No inhibitory effect on a-FP levels was observed with MeAla-6 (Table 2). On the other hand, the CsA-unrelated, immunosuppressive agent FK506 failed to reduce Epo levels in the medium, in comparison with control cultures (Fig 1).

Because it is known that the effects of CsA on target cells are mediated by specific cytoplasmic receptors, we searched for the presence of (a) CsA-receptor(s) in Hep3B cells. As shown in Fig 2, cytosolic proteins prepared from Hep3B cultures were found to bind $[^3H]$-CsA in a dose-related fashion, and the bound labeled molecule was efficiently displaced by the cold substrate. By hypothesizing a 1:1 stoichiometric interaction between $[^3H]$-CsA and the receptor(s), the calculated concentration of the CsA receptor(s) was 0.12 ± 0.04 µg/mg cytosolic proteins. To further characterize the CsA receptor(s), a molecular size-exclusion HPLC analysis of cytosolic extracts was performed, which allowed for the identification of a major peak of $[^3H]$-CsA binding protein corresponding to a calculated $M_r$ of about 18.0 Kd (Fig 3). The CsA-receptor protein was also identified by ligand-blotting experiments, in which gels were deliberately overloaded to allow evidention of even less represented CsA-binding species. However, only a single band was observed in these experimental conditions, with a $M_r$ of 17.5 Kd (Fig 3, insert).

To verify the hypothesis that the reduced levels of Epo found in the medium of cultures incubated with CsA were caused by an effect on the expression of the Epo gene, total RNA was prepared from CoCl$_2$-stimulated Hep3B cultures that had been incubated for 16 hours in the presence or absence of CsA. However, in four separate experiments, one of which is reported in Fig 4, we were unable to document any apparent reduction in the amount of Epo mRNA in cultures incubated with up to 1.6 µmol/L CsA. Then, we reasoned that alterations of posttranslational processes might be involved in causing the reduced Epo levels found...
in the medium of cultures incubated with CsA. To this end, the amount of Epo remaining within the cells at different times after stimulation with CoCl₂ was measured. While no effect was noticed within the first 4 hours of incubation, a progressive increase in the intracellular Epo content was observed in the presence of CsA (1.2 μmol/L) at 8 to 24 hours after CoCl₂ stimulation (Fig 5); at 24 hours, cells incubated with CsA contained about 10-fold the amount of controls’ Epo. The bulk (97% of total) of the intracellular Epo in cells incubated with CsA was found in the fraction precipitated at 100,000g and dissolved with Triton X-100, suggesting that it was membrane-associated (Table 3).

**DISCUSSION**

Results presented in this paper indicate that in Hep3B cultures incubated with CsA at nanomolar concentrations, the amount of Epo released in the medium is significantly reduced when compared with control cultures. The following considerations support the idea that the inhibitory effect of CsA was specific: (1) at the doses used, CsA did not cause a general reduction in total cellular protein synthetic rate, nor did it affect the secretion of α-FP, a constitutively secreted protein by Hep3B cells; (2) the immunosuppressive CsA analog MeAla-6 proved to be inhibitory, although

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**Table 3. Intracellular Distribution of Epo in Hep3B Cells**

<table>
<thead>
<tr>
<th>Subcellular Fraction</th>
<th>Control (EPO μU/mg protein)</th>
<th>CsA (EPO μU/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude nuclei</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Membrane-associated</td>
<td>5.5 ± 3.0</td>
<td>57.4 ± 10.0</td>
</tr>
<tr>
<td>Soluble</td>
<td>0.2 ± 0.2</td>
<td>1.7 ± 0.5</td>
</tr>
</tbody>
</table>

Hep3B cells were incubated in media containing 50 μmol/L cobalt chloride, in the presence or absence of CsA (1.2 μmol/L), and cells were collected 16 hours later. The subcellular fractionation was performed as described in Materials and Methods. Results are expressed as the mean ± SD from two experiments.

Abbreviation: NA, not measurable.
about 40% less efficiently on a molar basis than the parental drug. This correlates well with the previous demonstration that MeAla-6 binds to the CSA receptor, the protein cyclophilin, with an efficiency of about 50% in comparison with CsA and (3) the CSA-unrelated immunosuppressive agent FK506 had no effect on the release of Epo by Hep3B cells. We have also found that Hep3B cells contain a protein capable of binding [3H]-CsA, with a molecular weight comparable with the main cyclophilin species of T-lymphocytes (18.0 Kd). Finally, evidence has been provided that the reduced levels of Epo found in the culture medium of Hep3B cells are not caused by an effect of CsA on the expression of the Epo mRNA, at least at the level of sensitivity of Northern analysis; rather, they appeared to be dependent on (an) alteration(s) in the mechanisms leading to hormone secretion in the culture medium. In fact, the amount of Epo remaining inside the cells was significantly higher in cultures incubated with CsA than in controls, and subcellular fractionation studies indicated that the most part of the unsecreted, intracellular Epo was associated with the plasma membrane fraction.

The immunosuppressive activity of CsA results from the inhibition of some receptor-mediated signal transduction pathways in T lymphocytes. CsA becomes active after an alteration(s) in the mechanisms leading to hormone secretion in the culture medium. In fact, the amount of Epo remaining inside the cells was significantly higher in cultures incubated with CsA than in controls, and subcellular fractionation studies indicated that the most part of the unsecreted, intracellular Epo was associated with the plasma membrane fraction.

The results of our experiments seem to suggest that the formation of an active complex between CsA and the cyclophilin-like protein found in Hep3B cells may elicit some alterations in the posttranslational processing of Epo, with the consequent accumulation of the molecule within the endocellular membranes and a net reduction of the amount of hormone secreted. Theoretically, this might be caused by (1) a defect in the correct folding of the Epo molecule, because of the inhibition of PPlase activity, and/or (2) a defect in the intracellular translocation processes of the Epo molecules required for the secretion in the medium. About the first mechanism, it may be worthy to recall that Lodish and Kong observed that CsA inhibited the secretion of transferrin from another human hematoma cell line (HepG2) at concentrations not affecting the release of other proteins, and suggested this effect might be caused by a lag in the folding of transferrin molecules and to a retarded matura
tion from the endoplasmic reticulum (ER). However, experiments performed up to now failed to document significant structural alterations of the Epo molecule produced by Hep3B cells incubated with CsA (unpublished data, September 1992), possibly indicating that the first mechanism is less likely. Therefore, although the unequivocal intracellular localization of Epo in CsA-treated cultures will require the use of specific antibodies and further subcellular fractionation studies, at the moment we favor the second hypothesis, which has analogies with studies of a cyclophilin-like protein encoded by the ninaA gene of Drosophila melanogaster. The ninaA gene product, highly homologous to the B isoform of mammalian cyclophilin, is found only in the retina of Drosophila, and it has been shown to be essential for the posttranslational processing of rhodopsin Rh1 molecules, which require ninaA product for the exit from the ER and their traveling through the cytoplasm to the rhabdomeres. In fact, ninaA mutant flies showed a dramatic accumulation of Rh1 opsin molecules in the cisternae of ER. A similar mechanism of action has also been proposed by Hultsch et al., who observed that both CsA and FK-506 can inhibit receptor-mediated exocytosis of secretory granules from a rat basophilic leukemia cell line. They suggested that the two drugs might act by inhibiting the intracellular translocation of the secretory granules, in the same way as the translocation of nuclear factors required for the activation of the early genes involved in the immune response is affected in T lymphocytes.

Our proposed model is that the immunophilin found in Hep3B cells is physiologically involved in some steps of the intracellular processing and/or secretion of the Epo molecules, and that these processes are hindered in the presence of CsA. In this regard, it has been shown that a cyclophilin identified from a murine cDNA library (cyc P) binds specifically to a 77-Kd protein in the absence of the drug, while in the presence of CsA it binds to a 55-Kd protein. The authors suggested that the formation of the cyc P-77 complex may reflect the physiologic function of this immunophilin in the cells, while the cyc P-55 complex is involved in signal transduction events elicited by CsA binding. Furthermore, an immunophilin specific for FK-506 has been recently shown to associate, in the absence of the drug, with two heat-shock proteins and with the glucocorticoid receptor to form the inactive glucocorticoid receptor complex, thus suggesting a physiologic role for the FK506-binding protein different from that involved in immunosuppression.

In conclusion, we have observed that CsA is able to significantly affect the release of Epo in vitro by the Hep3B hematoma cell line, thus supporting previous suggestions that the reduced Epo levels characteristic of allogeneic BMT patients might be ascribed, at least in part, to the drug. Although it might be questioned whether Hep3B cells are a physiologic model of Epo production, the previous demonstration that CsA impairs Epo production in vivo seems to indicate that results obtained with the hematoma cell model are possibly applicable also to kidney cells, which represent the major source of the hormone in the intact animal. Finally, our data add some further insights into the roles of immunophilins in the regulation of protein metabolism in
cells other than T lymphocytes, and suggest the existence of a complex network of target molecules for CsA, whose knowledge might also help in understanding the molecular mechanisms of the toxicity associated with the drug.

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REFERENCES

17. Goldberg MA, Dunning SP, Bunn HF: Regulation of the erythropoietin gene: Evidence that the oxygen sensor is a heme protein. Science 242:1412, 1988
35. Friedman J, Weissman I: Two cytoplasmic candidates for immunophilin action are revealed by affinity for a new cyclophilin: One in the presence and one in the absence of CsA. Cell 66:799, 1991

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